Optimizing xeno-free culture conditions for human embryonic stem cells to correspond GMP-quality standards

Sarita Ketola
Master ‘s Thesis
University of Tampere
Institute of Medical Technology
April 2006

Acknowledgements

This study was carried out at the Institute for Regenerative Medicine Regea, University of Tampere. I would like to express my sincere gratitude to the whole staff of Regea and especially to Heli Skottman whose expertise and support have been crucial for the completion of this thesis. I also want to thank the staff of Histola Ltd and Kyllikki Haapala from Laboratoriokeskus Ltd for the fast and expert service. My thanks belong also to Heidi Koivisto and Matti Hakama for the help with the statistics and to Liisa Luoto for reviewing the language. Finally, I am very grateful to my family and friends for supporting me through this process.

Tampere, April 2006

Sarita Ketola

PRO GRADU –TUTKIELMA

Paikka:	TAMPEREEN YLIOPISTO Lääketieteellinen tiedekunta Lääketieteellisen teknologian instituutti Solu- ja kudosteknologia keskus, Regea
Tekijä:	KETOLA, SARITA PAULIINA
Otsikko:	Ihmisalkion kantasolujen eläinvapaiden viljelyolosuhteiden optimointi GMP laatuvaatimuksia vastaaviksi
Sivumäärä:	73
Ohjaaja:	FT Heli Skottman
Tarkastajat:	Professori Markku Kulomaa, FT Heli Skottman
Aika:	Huhtikuu 2006

Tiivistelmä

Ihmisalkion kantasolut (hES-solut) voivat tarjota ehtymättömän lähteen solusiirrännäisiin tulevaisuudessa. Nykyisissä viljelyolosuhteissa käytetään kuitenkin eläinperäisiä tuotteita, jotka aiheuttavat riskin eläinpatogeenien siirtymisestä ja solujen hylkimisestä ihmisen elimistössä. Lisäksi hES-solujen viljelyssä apuna tarvittavien tukisolujen työläs tuottaminen sekä kantasolujen hankala mekaaninen jakotapa tekevät hES-solujen suurten määrien tuottamisesta vaikeaa. Tutkimuksen ensimmäisenä tavoitteena oli optimoida hES-solujen viljelyolosuhteita kohti eläinvapaita olosuhteita testaamalla täysin eläinvapaita seeruminkorvikkeita (SR-3 ja X-Vivo 20) ja fibroblastien kasvutekijää (bFGF). Tutkimuksen muina tavoitteina oli löytää helpompi tapa hES-solujen jakamiseen sekä testata biomateriaalien soveltuvuutta korvata tukisolut hES-solujen viljelmissä.

Testauksen aikana hES-solujen erilaistumisaste pääteltiin ulkomuodon avulla ja vahvistettiin myöhemmin immunokemiallisesti käyttäen kantasoluspesifisiä markkereita erilaistumattomille soluille (AP, Oct-4 ja Nanog) ja erilaistuneille soluille (SSEA-1). Testatuilla SR-3 ja X-Vivo 20 menetelmillä kasvatetut hES-solut olivat täysin erilaistuneita, kun taas osa testi bFGF:n kanssa kasvatetuista soluista oli erilaistumattomia. Näiden solujen lukumäärä oli kuitenkin hyvin alhainen. Eläinvapaalla entsyymillä, Tryplellä, jaetut hES-solut olivat enimmäkseen erilaistumattomia ja niiden lukumäärä oli samankaltainen kontrollin kanssa. Lisäksi solujen karyotyyppi oli normaali 46,XX 31 jakokerran jälkeen. Näistä hES-soluista erilaistetut soluaggregaatit, ns. embryoid bodit, ilmensivät eri alkion kerroksille yleisiä geenejä ja proteiineja käänteiskopioija-PCR:n ja immunokemiallisten analyysien perusteella, osoittaen hES-solut pluripotenteiksi. Testatut biomateriaalit mahdollistivat hES-solujen kiinnittymisen kyllästetyssä kasvatusliuoksessa, mutta solujen erilaistumatonta kasvua ei pystytty osoittamaan. Solut, jotka siirrettiin takaisin tukisolujen päälle, olivat kuitenkin enimmäkseen erilaistuneita

Testatut SR-3, X-Vivo 20 ja bFGF sekä biomateriaalit eivät ylläpitäneet hES-solujen erilaistumatonta kasvua paremmin kuin kontrolliolosuhteet, mutta testattu Tryple-jakotapa ylläpiti solujen kasvua hyvin. Vaikka tämä ei ollut nopeampi tapa kuin mekaaninen jako, se helpottaa ohuiden kantasolukolonioiden jakamista. Tryple-jakotavalla koloniat voidaan paloitella pienempiin paloihin kuin mekaanisella jaolla, ja näin saadaan aikaan hES-solumäärän nopeampi monistuminen.

MASTER'S THESIS

Place:	UNIVERSITY OF TAMPERE Faculty of Medicine Institute of Medical Technology (IMT) Institute for Regenerative Medicine Regea
Author:	KETOLA, SARITA PAULIINA
Title:	Optimizing xeno-free culture conditions for human embryonic stem cells to correspond GMP-quality standards
Pages:	73
Supervisor:	Dr. Heli Skottman
Reviewers:	Professor Markku Kulomaa, Dr. Heli Skottman
Date:	April 2006

Abstract

Human embryonic stem cells (hESCs) can offer an unlimited cell source for cellular transplantation, but the currently used culture conditions contain animal-derived products that bear a risk of transmitting animal pathogens and causing a rejection when cells are transplanted. In addition, the laborious production of feeders needed for hESC culture and the difficult mechanical passaging of hESCs make the large-scale production of hESCs difficult. The first aim of this study was to optimize culture conditions for hESCs towards animal-free conditions by testing totally animal-free serum replacements (SR-3 and X-Vivo 20) and basic fibroblast growth factor (bFGF). Other aims of the study were to find an easier way for hESC passaging and to test the possibility of growing hESCs without feeder cells using biomaterials.

The differentiation stage of the hESC colonies was determined during the testing by morphology and later confirmed with immunochemistry using markers specific for undifferentiated hESCs (AP, Oct-4 and Nanog) and for differentiated hESCs (SSEA-1). The hESCs grown with the SR-3 and X-Vivo 20 were all differentiated where as some of the hESCs grown with the test bFGF were undifferentiated. However, the amount of these cells was very low. The hESCs passaged with animal-free enzyme Tryple were mostly undifferentiated and the amount of these cells was similar with control. In addition, the cells had normal 46,XX karyotype after 31 passages. The embryoid bodies (EBs) differentiated from these hESCs expressed genes and proteins common to the three embryonic germ layers based on the reverse transcriptase-PCR and immunochemistry analysis, showing the pluripotency of the cells. The tested biomaterials allowed the attachment of hESCs in the presence of conditioned medium, but the undifferentiated growth of hESCs could not be determined. However, the cells plated back on feeders after one passage on biomaterials, were mostly differentiated.

The tested SR-3, X-Vivo 20 and bFGF as well as the biomaterials were not able to maintain the undifferentiated growth of hESCs better than the control conditions, but the Tryple passaging method supported the growth of hESCs well. Although it was not faster way than mechanical passaging, it makes the passaging of thin hESC colonies easier and the colonies could be split into smaller pieces than with mechanical passaging, resulting in faster expanded growth of hESCs.

Table of Contents

Abbreviations

AFP	Alphafetoprotein
AFT	Adult fallobian tube
ANOVA	Analysis of variance
AP	Alkaline phosphatase
ATCC	American type culture collection
α-sr-1	Alpha-sarcomeric actin
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BSA	Bovine serum albumine
CM	Conditioned medium
DAPI	4´,6-Diamidino-2-phenylindole
EB	Embryoid body
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FISH	Fluorescence *in situ* hybridization
hFF	Human foreskin fibroblast
FGFR	Fibroblast growth factor receptor
GADPH	Glyseraldehyde 3-phosphate dehydrogenase
G-banding	Giemsa-banding
GMP	Good manufacturing practice
hESC	Human embryonic stem cell
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
hrbFGF	Human recombinant basic fibroblast growth factor
HSA	Human serum albumin
hTERT	Human telomerase reverse transcriptase
ICM	Inner cell mass
Ig	Immunoglobulin
IVF	*In vitro* fertilization
Ko-SR	Knock-out serum replacement
LIF	Leukemia inhibitory factor

MEF	Mouse embryonic fibroblast
MHC	Major histocompatibility complex
mHC	Minor histocompatibility complex
MuLVs	Murine leukemia viruses
ND-1	NeuroD-1
NF-68KD	Neurofilament 68 kilodaltons
PB	Phosphate buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RT	Reverse transcriptase
SCID	Severe combined immunodeficient
SCNT	Somatic cell nuclear transfer
SR	Serum replacement
SSEA	Stage specific embryonic antigen
TGF	Transforming growth factor
TiO2	Titania
Ti	Titanium
TRA	Tumor related antigen
UM	Unconditioned medium
Zr	Zirkonium

1. Introduction

Human embryonic stem cells (hESCs) have unique proliferation and differentiation capacities, which makes them a valuable source for cell transplantation and tissue engineering. Many have reported their ability to differentiate into a variety of specific cell types, including neurons, cardiomyocytes and insulin secreting cells (Lumelsky *et al.*, 2001; Passier *et al.*, 2005; Schulz *et al.*, 2003). Human ESC-derived tissue-specific cells may have therapeutic potential for the treatment of Parkinson's disease, spinal cord injury, heart disease, diabetes and other degenerative conditions.

Thomson *et al.* were the first to derive hESCs from the inner cell mass (ICM) of a blastocyst gained after *in vitro* fertilization (IVF) treatment and to culture them as permanent cell lines (Thomson *et al.*, 1998). They used culture media containing fetal bovine serum (FBS) and mitotically inactivated mouse embryonic fibroblasts (MEFs) as feeder cells, which are believed to secrete factors into the medium and provide the necessary attachment for the undifferentiated growth of hESCs. However, animal-derived components, nonhuman sera, and animal feeder cells in the cultures bear a risk of transmitting animal pathogens and animal proteins to hESCs (Amit *et al.*, 2005; Martin *et al.*, 2005). These components need to be removed from the culture in order to use the cells in transplantations for humans because they enhance the risk of immune rejection and zoonosis in cell transplantation recipients.

A feeder-free culture system has been developed using Matrigel or laminin coated surfaces with conditioned media collected from cultures of mouse feeder cells (Xu *et al.*, 2001). This system eliminates the direct contact with MEFs, but it is still not animal-free. Numerous human feeders including human foreskin fibroblasts (hFFs) (Amit *et al.*, 2003; Hovatta *et al.*, 2003) and human adult marrow cells (Cheng *et al.*, 2003) have been shown to support hESC growth, but the hESC culture media used in these studies contains animal-derived proteins. Human serum has been used instead of FBS in the hESC culture medium (Richards *et al*., 2002), but prolonged use of human serum leads to increased differentiation rates of hESCs (Koivisto *et al.*, 2004; Richards *et al.*, 2003).

Amit *et al.* described serum-free culture conditions for hESCs using serum replacement (SR) in hESC culture medium and postnatal human fibroblasts as feeder cells, but the used cell lines were originally derived using FBS and MEFs (Amit *et al.*, 2003). The derivation of hESCs on hFFs and in SR has now been reported (Inzunza *et*

al., 2005). The addition of basic fibroblast growth factor (bFGF) in this culture system is required (Koivisto *et al.*, 2004). Although this was a step forward, the SR and the bFGF used still contain animal-derived products. The SR form Gibco Invitrogen contains bovine serum albumine (BSA) and bovine insulin. In addition the human recombinant basic FGF (hrbFGF) from R & D Systems contains BSA as a preservative.

The passaging of hESCs is performed by enzymatic treatments (trypsin, dispase or collagenase IV) or by mechanical dissection. Mechanical dissection is laborious, demands skills, and is time-consuming making the rapid expansion of hESCs hard if not impossible. The enzymes used for hESC passaging are often animal-derived and the use of enzymes can promote chromosomal aneuploidy, especially trisomy 12 and/or 17 (Draper *et al*., 2004b; Mitalipova *et al.*, 2005).

In the present study, the specific aim is to optimize culture conditions for hESCs towards animal-free conditions by testing an animal-free serum replacement that is added to the culture medium, an animal-free ready-made serum replacement medium and hrbFGF without BSA. Another aim of the study is to improve the passaging technique of hESCs using animal-free recombinant enzyme. The third aim is to test the possibility of growing hESCs without feeder cells using biomaterials.

2. Review of the literature

2.1. Stem Cell

Stem cell is a unique cell that can differentiate into many kind of cells found in the body. It can also proliferate as a stem cell, and in theory it can be cultured *in vitro* indefinitely (Figure 1). Stem cells can be found in embryo, fetus and some adult tissues.

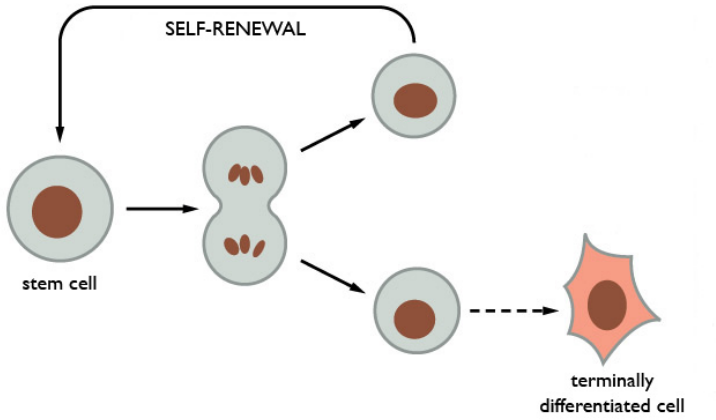


Figure 1. *A stem cell. Each daughter cell can either remain a stem cell or differentiate into a specific cell usually through additional cell divisions. (Alberts et al., 2002).*

Stem cells can also be categorized according to their differentiation potential. Totipotent cells have unlimited potential because they are capable of forming an entire organism. Fertilized oocyte is a totipotent cell and it creates identical totipotent cells with each cell division. By the fourth day, the totipotent cells begin to specialize, forming a blastocyst. The outer layer of the cells (trophectoderm) in the blastocyst forms the placenta and other necessary tissues in the uterus required for the fetus to develop. The inner cell mass (ICM) of the blastocyst cannot form an entire organism anymore, but they can form virtually every type of tissues in the body and are therefore referred to as pluripotent cells. The pluripotent cells then further specialize into another type of stem cells, multipotent stem cells. Multipotent stem cells are committed to give rise to cells that have a particular function, for example, blood stem cells give rise to red blood cells, white blood cells and platelets, and skin stem cells give rise to the different types of skin cells. These cells are considered committed stem cells or progenitor stem cells.

2.1.1. Embryonic stem cells

The first human embryonic stem cell (hESC) line was derived in the Thomson laboratory at the University of Wisconsin (Thomson *et al.*, 1998). Since then over a 250 new hESC lines have been derived in the world. Human ESCs are derived from preimplantation stage embryos, a process which involves culturing embryos to the blastocyst stage. Embryos are donated by infertile couples undergoing *in vitro* fertilization (IVF) treatment. The quality of the embryos is determined according to their morphology and only good quality embryos are used in the infertility treatments. Excess or poor quality embryos that would otherwise be discarded are used in stem cell line derivation. The donated cleavage stage embryos (usually day 2 after fertilization) are cultured to the blastocyst stage. The zona pellucida surrounding the blastocyst is removed enzymatically using pronase. Then the trophectoderm is removed by immunosurgery using rabbit antihuman whole serum and guinea pig complement serum (Hovatta *et al.*, 2003). The remaining ICM is then transferred onto specific cell layer, a feeder cell layer, and allowed to proliferate. The undifferentiated cells, judged by morphology, are then chosen for each further cell splitting called passage. The derived cell line consists of embryonic stem cells that can proliferate and replace themselves indefinitely.

In natural conditions, *in vivo*, the ICM differentiates into generate primitive ectoderm, which ultimately differentiates into the three embryonic germ layers (endoderm, mesoderm and ectoderm) (Odorico *et al.*, 2001). *In vitro* cultured hESCs have also the potential to differentiate into these germ layers. See Figure 2.

Human ESCs are pluripotent cells and their essential characteristics include (1) derivation from the preimplantation embryo, (2) prolonged undifferentiated proliferation, and (3) stable developmental potential to form derivatives of all three embryonic germ layers even after prolonged culture (Thomson *et al.*, 1998). In addition, hESCs maintain a normal euploid karyotype over extended culture, express high levels of Oct4 and Nanog, and show telomerase activity (Hoffman and Carpenter, 2005). Transcription factor Oct4 and its target gene Nanog are believed to take part in regulating the pluripotency of hESCs (Chambers *et al.*, 2003; Nichols *et al.*, 1998) and the expression of telomerase is highly correlated with immortality in human cell lines (Kim *et al.*, 1994).

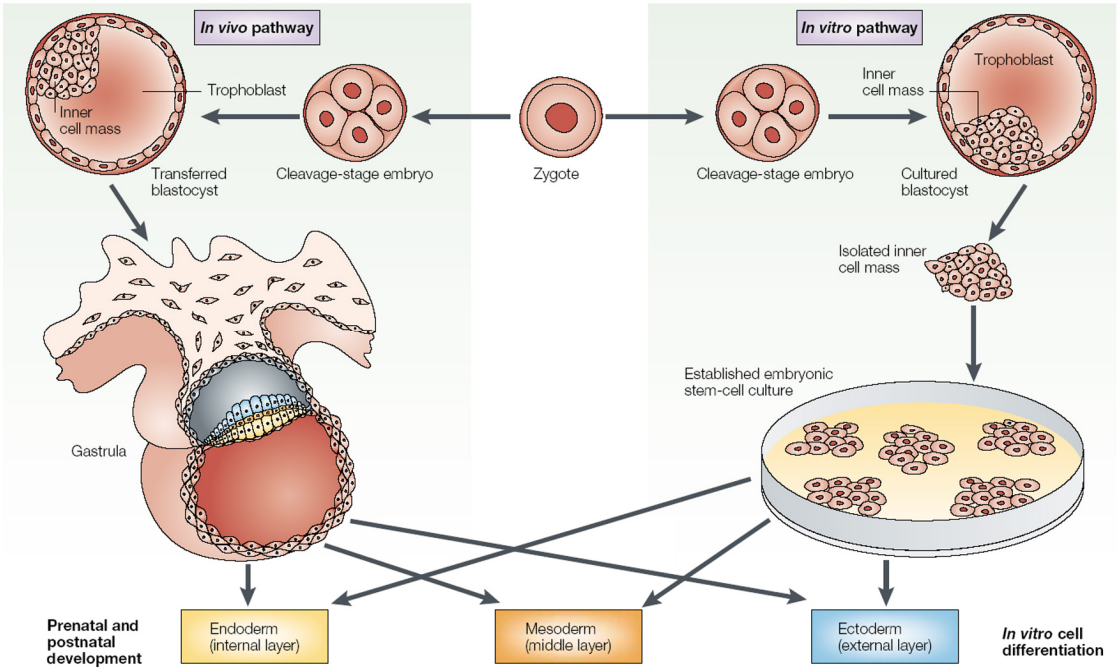


Figure 2. *Two fates of an in vitro fertilized zygote. The zygote is usually cultured 2-3 days, after which it is transplanted into a uterus where it will develop into a human being. Alternatively the zygote can be cultured to blastocyst and the inner cell mass of the blastocyst can be isolated and cultured in vitro as stem cells. These cells can then be differentiated into cells representing all three embryonic germ layers. (Figure modified from Bradley et al. 2002).*

2.1.2. Adult stem cells

When the embryo develops, the cells begin to differentiate and to lose their developmental potential; nevertheless, pools of stem cells persist in several adult tissues. These multipotent cells have been found in various tissues, including bone marrow, peripheral blood, brain, liver and reproductive organs. These cells are maintained locally or by the systemic circulation and are activated by environmental stimuli for tissue regeneration (Asahara *et al.*, 2000).

The differentiation potential of adult stem cells was previously though to be strictly limited to cell lineages found within the tissue of origin. However, today this view has been challenged. Several studies with mice have proposed that bone-marrow-derived cells could give rise to muscle cells (Ferrari *et al.*, 1998; Orlic *et al.*, 2001). Furthermore, neural stem cells have proved to differentiate into many cell types including hepatocytes, cardiomyocytes and epidermal cells (representing all three embryonic germ layers) (Clarke *et al*., 2000). In these studies the stem cells have been exposed to an environment that they typically would not encounter, which indicates that a stem cell may receive differentiation signals from neighboring cells. This might be the reason

why adult stem cells have previously seemed to generate only the types of cells present in a specific tissue (Clarke and Frisen, 2001). In a new environment, the stem cells could be able to respond to the signals, which would reprogram the stem cells to generate cells appropriate for the new environment (Frisen, 2002). This is known as stem cell plasticity. However, recent studies have suggested alternative explanations to stem cell plasticity. The experimental situations are often artificial and may be far from the physiological situation and often there has been damage to the tissue, which may be a perquisite for the observed effects (Frisen, 2002). Stem cell plasticity may also be the result of cell fusion, where adult somatic cells can gain differentiation potential by fusion with less differentiated cells (Terada *et al.*, 2002; Ying *et al.*, 2002). However the fact is none of the studies suggesting plasticity have excluded all alternative explanations.

2.2. Future possibilities of hESCs

Human ESCs provide a lot of new information and hope for regenerative medicine. In addition, they offer a new model for understanding human development and therefore can help us to understand abnormal development. Experimental manipulation and functional studies of the post-implantation human embryo are ethically unacceptable. What is known about early human development so far is based on histological sections of a few human embryos and on analogy to experimental embryological studies of the mouse (Thomson and Odorico, 2000). Because the earliest events of human development are critically involved in human infertility, pregnancy loss and birth defects, hESCs could provide answers to these problems.

The hESCs can also be used to study the function of genes. In mouse, most gene function studies require the generation of transgenic or gene knockout animals. Human ESCs could provide *in vitro* approach that lacks the limitations of *in vivo* studies in mouse, for example the required long time, the relatively small-scale research and the need for animal testing. Human ESC lines that lack a specific gene or over express one can be created, and the influence of manipulation on the normal growth and development of specific tissues can be studied (Keller and Snodgrass, 1999).

Human ESCs also provide valuable test system for evaluating the toxicity and efficacy of new medicines or chemicals. Currently used animal models provide us with important information about how the chemical acts *in vivo*, but they cannot always

predict the effects on human cells. Cultures of other human cells are also used for this purpose, but the cell lines have usually been cultured *in vitro* for long periods, which may have changed their characteristics compared to the *in vivo* cells. Human ESCs could be differentiated into specific cell types, which would mimic the *in vivo* response of the cells or tissue to the drug or toxin better than the long-term cell lines. The hESCs could be used in pharmaceutical, cosmeceutical and agrochemical industries instead of animal testing and they could offer safer, and potentially cheaper models for drug and chemical screening (Rolletschek *et al.*, 2004; Seiler *et al.*, 2004).

The most obvious application of hESCs is in cell-replacement therapies. At the moment, many diseases can be treated only by a transplant of tissue or organ, but in some cases the need for donated organs could be reduced or eliminated by using cell therapies. Cell transplantation could offer a treatment also for diseases that are currently untreatable, such as spinal cord injury. Studies with several animal models suggest that differentiated derivatives of ESCs could be used in cell transplantations (Paul *et al.*, 2002). Human ESCs have been differentiated into several different cell types, such as neurons, cardiomyocytes, oligodendrocytes, and insulin secreting cells, suggesting that hESCs could be used in the future as a treatment for Parkinsson's disease, heart failure, spinal cord injury and diabetes, among others (Faulkner and Keirstead, 2005; Lumelsky *et al.*, 2001; Nistor *et al.*, 2005; Passier *et al.*, 2005; Schulz *et al.*, 2003)

Before any of these applications can be used properly, the differentiation of hESCs into specific cell types must occur efficiently in defined conditions. If undifferentiated cells are transplanted the environmental signals will strongly influence on the differentiation of the cells. These signals are difficult or even impossible to predict and the transplant may form a teratoma. The hESCs can be directed to differentiation pathways *in vitro* by changing their culture conditions. Using End-2 cells and serum replacement (SR) Passier *et al.* were able to produce cardiomyocytes, and using MedII-conditioned medium from Hepa2 cells Shulz *et al.* differentiated hESCs into neurons (Passier *et al.*, 2005; Schulz *et al.*, 2003). Insulin secreting structures have been able to form by supplementing basic fibroblast growth factor (bFGF) and nicotinamide into the growth medium and oligodendrocytes by using a combination of different hormones and growth factors (Lumelsky *et al.*, 2001; Nistor *et al.*, 2005). The effect of growth factors in the early differentiation of hESCs has been studied. Shuldiner *et al.* were able to alter the relative proportions of specific cell types with certain growth factors, but not to exclusively direct the differentiation into only one cell type (Schuldiner *et al.*, 2000).

The effect of growth factors is highly dependable on the molecular signature of the cell, what kind of ligands and receptors the cell is producing. The molecular signature of undifferentiated hESCs and their differentiated derivatives have been compared and 918 genes have been found to be enriched in the undifferentiated cells (Sato *et al.*, 2003). These include ligand/receptor pairs and secreted inhibitors of the FGF, transforming growth factor-β/bone morphogenetic protein (TGFβ/BMP), and Wnt pathways. It is essential to the expanded culture of the hESCs to define the factors maintaining the pluripotency of the cells and it could also help to found the routes to the differentiation pathways. Skottman *et al.* compared the gene expression profiles of seven genetically independent hESC lines with those of nonlineage-differentiated cells derived from each line (Skottman *et al.*, 2005). They found 8,464 transcripts expressed in all hESC lines, from which 280 genes were specific for hESCs and 219 genes were differentially expressed in all hESC lines compared with nonlineage-differentiated cells. The expression profiles of the seven hESC lines were similar, but there were some variation reflecting their genetic differences. This variation could influence on the preferential differentiation potential of the hESC lines. However, from the 8,464 transcripts found, more than 45 % have no yet-known biological function, indicating that a large number of factors influencing the hESC pluripotency are yet to be determined.

2.3. Culture of hESCs

2.3.1. Feeder cells

The first hESC line was derived and cultured on mouse embryonic fibroblast (MEF) cell layer using 20 % fetal bovine serum (FBS) in the hESC culture medium (Thomson *et al.*, 1998). The fibroblasts were gamma irradiated to obtain mitotically inactive cells that do not proliferate. These so-called feeder cells enable hESCs to remain undifferentiated. The exact mechanism is not known, but the feeder cells are believed to secrete factors into the medium that conjugate with the extra cellular matrix (ECM) or interact with membrane bound proteins. They also provide attachment surface for hESCs that is essential for the undifferentiated growth of hESCs. Proliferative MEFs with certain densities are also able to support the growth of hESCs (Xie *et al.*, 2005) but it seems unnecessary to use feeder cells that also themselves demand space to grow. These culture conditions, however, expose the hESCs to xenogeneic components. If the cells are ever to be used in cell replacement therapies, these xenogeneic components cause a

risk of infection by nonhuman pathogens and cell rejection by animal proteins on hESC surface. Mice harbor a variety of parasites, bacteria, and viruses potentially pathogenic for humans. Among these, endogenous retroviruses, including murine leukemia viruses (MuLVs) are able to infect human cells (Amit *et al.*, 2005). Although Amit *et al.* did not found any evidence for infection of hESCs by MEF origin MuLV, it is a matter of great concern. Beside the feeder layer, also the medium used for the derivation and culture of feeder cells contains high quantities of animal-derived products, usually FBS. MEFs and animal-derived medium components contain the nonhuman sialic acid Neu5Gc which hESCs can incorporate (Martin *et al.*, 2005). This could lead to an immune response mediated by natural antibodies to Neu5Gc present in most humans (Martin *et al.*, 2005). The amount of Neu5Gc could be minimized by using human feeder cells and human serum or SR in the culture of hESCs.

Richards *et al*. demonstrated first that human feeders support the undifferentiated growth of hESCs (Richards *et al.*, 2002). They used human adult fallobian tubal (AFT) epithelial feeder layer, human fetal skin and fetal muscle feeder layers successfully. The use of AFT feeder layer would be more convenient because the derivation of human fetal cell lines from human abortuses may cause ethical concerns. Although human feeders reduce the amount of animal pathogens, Richards *et al.* used FBS in the feeder culture medium as well as in hESC culture medium exposing the hESCs still to animal-derived components.

Amit *et al.* managed to derive human foreskin fibroblast (hFF) feeders in human serum and in SR (Amit *et al.*, 2003). They found no differences in their growth rates and morphologies compared to feeders grown in FBS. These feeder cells supported the undifferentiated growth of hESCs equally well as feeders derived in FBS. However, no other group has yet reported the derivation of feeder cells in SR, indicating that there might be some problems with the method. Amit *et al.* cultured hESCs on these feeder cells using culture medium containing 15 % SR instead of FBS. Although they managed to remove the main animal-derived components from the culture of hESCs, the used hESCs were still originally derived using FBS and MEFs.

Human FFs have been used also in the derivation of new hESC lines (Hovatta *et al.*, 2003). These feeder cells were commercially available from American type culture collection (ATCC), which reduces the work amount greatly in hESC culture. The medium used in the culture of both feeder cells and derived hESCs contained FBS, but the same group announced later the derivation of new hESC lines using the same commer-

cially available feeders and hESC culture medium containing SR (Inzunza *et al.*, 2005). The culture medium of the feeder cells were changed to medium containing SR shortly after their mitotic inactivation, but the derivation and growth of feeders still required FBS in the culture medium.

Various other human feeders have also been evaluated showing that adult skin and commercial fetal skin feeders maintain the undifferentiated growth of hESCs (Richards *et al.*, 2003). Human marrow derived fibroblastic stromal cells have also the capacity to maintain the growth of undifferentiated hESCs in SR containing medium (Cheng *et al.*, 2003). However, not all human feeder cells support hESC growth equally well, for example human adult muscle cells (Richards *et al.*, 2003).

2.3.2. Feeder-free culture of hESCs

In addition to different feeder cells, various coatings of culture plates have been tested for the culture of hESCs. One tested coating is commercially available Matrigel, which has a similar structure with the ECM. It is a soluble basement membrane extract of the Engelbreth-Holm-Swarm mouse tumor, and consists of laminin, collagen IV, entactin, heparan sulfate proteoglygan and various growth factors (Hoffman and Carpenter, 2005). Although Matrigel might simplify the handling of hESC cultures, it is derived from mouse and cannot be used in clinical grade hESC cultures. Laminin, collagen IV, and fibronectin alone have also been tested for the hESC culture, where laminin showed to maintain the hESC growth equally well as Matrigel (Xu *et al.*, 2001). Culture plates coated with human serum have also been reported to maintain the undifferentiated growth of hESC (Stojkovic *et al.*, 2005). This coating has similar surface structure than Matrigel has, indicating that the three-dimensional structure may also be important.

An optimal alternative to feeders and coated matrixes would be the use of a bio-material. Three-dimensional structures have already gained an interest in the field of stem cell differentiation. Networks made from different concentrations of collagen I fibers and fibronectin or laminin have been tested for mouse embryoid body (EB) differentiation from mouse ESCs (Battista *et al.*, 2005). They concluded that both the composition and strength of the supportive matrix played an important role in EBs development. Also human EBs have been differentiated from hESCs on three-dimensional scaffolds made out of poly(lactic-co-glycolic acid), poly(L-lactic acid) and porous alginate (Gerecht-Nir *et al.*, 2004; Levenberg *et al.*, 2003). The use of polymeric scaffolds in hESC differentiation holds a promise of engineering tissues and organs for

transplantation therapies. Biomaterials could be useful also to the undifferentiated growth of hESCs. With right kind of surface structure in combination with different growth factors, biomaterials could be used in steady quality large-scale culture of hESCs without feeder cells. Testing the biomaterials used in implants could be the first step. Titania coated titanium implants, for example, have good adherence to soft tissue (Areva *et al.*, 2004). If this nonresorbable, reactive titania would be used to coat a culture dish, it could allow hESCs to attach to it. Considering that the attachment of the hESCs is crucial for their undifferentiated growth, this coating together with appropriate culture medium and growth factors could provide a promising culture condition for hESCs.

2.3.3. Human ESC culture medium

The animal-derived components must be removed also from the culture medium of hESCs. By now, almost all research groups have replaced the FBS from the hESC culture medium by SR. Amit *et al.* reported the use of SR first, and others have followed (Amit *et al.*, 2003). However, the most commonly used SR (from Gibco Invitrogen) contains animal-derived components, such as BSA, although in much less quantities than in FBS (Hoffman and Carpenter, 2005). Even a small amount of BSA in culture medium can cause the hESCs to incorporate Neu5Gc from the medium, which could cause an immune rejection if the cells were used in transplantation (Martin *et al.*, 2005). The amount of Neu5Gc could be minimized by using human serum or SR with only human orthologs or recombinant proteins. The use of human serum has been tested and the derivation of new hESC line in human serum containing medium and on human fetal feeders have been successful (Richards *et al.*, 2002). However, the prolonged use of human serum in hESC culture medium leads to increased differentiation rates of hESCs (Koivisto *et al*., 2004; Richards *et al.*, 2002; Richards *et al.*, 2003).

Different conditioned media (CM) that are collected from feeder culture plates after 24 hours of incubation have been tested for the feeder-free culture of hESCs. Xu *et al*. reported first the use of Matrigel and MEF-CM in hESC culture (Xu *et al.*, 2001). CM from human fetal and adult fibroblasts have also been tested together with Matrigel, human ECM, collagen I and laminin coatings, but these combinations did not support the undifferentiated growth of hESCs (Richards *et al.*, 2002). The CM from one human source has been able to support the hESC growth cultured on Matrigel. Xu *et al.* derived fibroblast-like cells from hESCs and made them immortal by infecting the cells with a

retrovirus expressing human telomerase reverse transcriptase (hTERT) (Xu *et al.*, 2004). The CM from these cells supports the hESC growth under feeder-free conditions. The same CM was later used successfully with human serum matrix coating instead of Matrigel reducing the exposure of animal ingredients significantly (Stojkovic *et al.*, 2005). However, no other group has yet reported similar results.

The use of CM still requires the laborious culture of feeder cells. Few groups have been able to culture hESCs without feeder cells and even without CM. Human ESCs can be cultured on Matrigel with unconditioned medium (UM) if the cells are plated at high densities (Draper *et al*., 2004a). Draper *et al.* showed also that laminin with fibronectin and/or collagen IV coatings supports the undifferentiated growth of hESCs in UM. However, the information about the serum used in the medium is not given. In addition, the culture of hESCs on feeder cells seems to support the hESC growth better than these coatings. Commercially available serum free medium (X-Vivo 10) that contains only human sourced recombinant proteins and growth factors also supports the growth of hESCs with Matrigel and human laminin coated surfaces (Li *et al.*, 2005). However, the feeder-free culture conditions of hESCs are usually strongly dependable on high concentrations of exogenously added growth factors.

The various reported culture conditions are difficult to compare, because each group has used different base media, matrix or feeders, cell lines and cell passage numbers (one splitting equals one passage). Although hESCs have been shown to grow in each of these conditions, it is unclear which, if any, of the culture conditions is the optimal one. The use of single substrate for hESC growth is desirable, but the substrates used are still undefined components, and may have a lot-to-lot variability (Hoffman and Carpenter, 2005). It is also unclear whether the hESCs maintained in these different substrates are equivalent. In fact, the gene expression signature of hESCs is reported to be different when cultured with SR and FBS (Skottman *et al.*, 2006). Skottman *et al.* found 1471 differentially expressed genes from which 470 genes were up-regulated in cells cultured in SR. These were genes that were mainly involved in signaling, development, and cell proliferation, which could explain the increased growth rate of undifferentiated cells in SR containing culture conditions compared to FBS (Koivisto *et al.*, 2004; Skottman *et al.*, 2006).

2.3.4. Basic fibroblast growth factor

Many growth factors have been implicated as regulators of hESC self-renewal, including the members of transforming growth factor-β1/bone morphogenetic protein (TGF-β1/BMP) superfamily, fibroblast growth factor (FGF) family and Wnt family. Combinations of these growth factors or some of them alone have been reported to be important for maintaining the undifferentiated growth of hESCs. Amit *et al.* presented a feeder and serum free culture system based on fibronectin matrix and culture medium containing SR together with TGF-β1, basic FGF (bFGF) and with or without leukemia inhibitory factor (LIF) (Amit *et al.*, 2004). Although it was left uncertain how LIF influences the cells in this system it has been shown previously that LIF alone does not maintain the undifferentiated self-renewal of hESCs (Thomson *et al.*, 1998). Basic FGF has also been used in combination with noggin, a BMP-antagonist. SR contains BMP-like activity, which can result to hESC differentiation (Xu *et al*., 2005). This activity can be reduced by noggin and bFGF or by high concentration of bFGF alone which allows the undifferentiated proliferation of hESCs in the absence of fibroblasts or CM (Xu *et al.*, 2005). It has been shown that the hESCs grown on feeder cells also required the addition of bFGF, but only in serum-free conditions (Amit *et al.*, 2000; Koivisto *et al.*, 2004). The concentrations of bFGF used are usually between 4 to 10 ng/ml, but in feeder-free conditions the concentration used can be as high as 100 ng/ml.

Levenstein *et al.* have tested the effects of several different concentrations of bFGF using UM and Matrigel (Levenstein *et al.*, 2006). 4, 24 and 40 ng/ml bFGF failed to support hESC culture, but 100 ng/ml and 250 ng/ml were capable of sustaining undifferentiated hESC proliferation with effectiveness comparable to CM. The need for high concentration of bFGF in UM may result from the faster degradation of bFGF in UM than in CM. Fibroblasts and fibroblast CM may sustain hESCs in part by secreting either protease inhibitors or binding proteins that modulate bFGF stability (Levenstein *et al.*, 2006).

The ligand/receptor pairs of the FGF signaling pathway have shown to be enriched in the undifferentiated hESCs (Sato *et al.*, 2003). These components of the pathway suggests that endogenous as well as exogenous FGF signaling may play an essential role in the undifferentiated state. There are five isoforms of endogenously produced bFGF in humans. The low-molecular isoform (18 kDa) is released from the cell, bound to transmembrane receptors and believed to regulate cell proliferation and differentia-

tion in autocrine or paracrine way. Human ESCs are found to secrete this isoform and can so activate the FGF receptors in an autocrine manner. Dvorak *et al.* suggests that all of these isoforms are similarly expressed in both undifferentiated and differentiated hESCs, but that the expression pattern of FGF receptors is changed. According to Dvorak *et al.* the four FGF receptor kinases, named FGFR1 through FGFR4, have the following expression order in undifferentiated hESCs: FGFR1 → FGFR3 → FGFR4 → FGFR2, with FGFR1 being most abundant. In differentiated cells, the expression pattern is changed to FGFR1 → FGFR4 → FGFR3 → FGFR2 and the expression of all the receptors is dramatically elevated, suggesting that the relative levels of FGFR expression, rather than bFGF expression, is coupled to conditions that direct hESCs to differentiate. (Dvorak *et al.*, 2005)

Despite the common use of bFGF in hESC culture the function of bFGF is poorly defined. Kim *et al.* proposed that bFGF helps maintain self-renewal of hESCs through activation of the PI3K/Akt/PKB pathway (Kim *et al.*, 2005). They revealed that if bFGF is removed from the media, the phosphorylation of the Akt/PKB molecule is decreased and the expressions of ECM molecules such as collagen type IV α1, laminin α1 and laminin receptor are nearly eliminated. These above studies highlight the important role of bFGF in the hESC culture.

2.3.5. Passaging of hESCs

The adherence of hESC to maintain their cell-cell interactions is important for their undifferentiated proliferation. The hESCs were originally passaged as small clumps, because of the previous reports of poor plating efficiency of single cell nonhuman primate ESCs (Thomson *et al.*, 1995). The derivation of hESCs as cell clusters from ICM, however, results in heterogenous cell populations. Further passaging by clumps maintains the mixed cell populations, and it cannot be excluded that these populations consist of multiple precursors or committed stem cells that together differentiate to all embryonic germ layers instead of one cell. Homogenous hESC line should be created to proof the pluripotency of stem cells. The clonal derivation of hESCs from existing cell lines has been reported (Amit *et al*., 2000; Heins *et al.*, 2006). The hESCs were dissociated into single cells by ethylenediaminetetraacetic acid (EDTA). The cells were then transferred to individual culture wells using micropipette under microscope. The resulted cell lines were then tested to be stem cells and the pluripotency of the cells was shown. The cloning efficiency, however, was poor and only two groups have reported

the successful cloning of hESC lines, indicating that the methods must be further developed.

Whether the cell line is clonal or not, it must be passaged in clumps. There are two different ways to do this, mechanical or enzymatic. Mechanical passaging is performed by splicing the colonies under a microscope, detaching the pieces from the plate and then transferring them onto fresh feeder plates. This method is time consuming and it demands skills, due to which it is difficult to process many cells at a time. Enzymatic method is faster and simpler. The cells are exposed to enzyme, which dissociates the cells. The dissociation is usually further improved by pipetting the cells gently. Typical reagents for this purpose include collagenase IV, trypsin and dispase. Some nonenzymatic solutions are also available, such as cell dissociation buffer. Some of these reagents are, however, xenogeneic proteins, for example trypsin is usually derived from porcine pancreas. These reagents are not recommended to use for hESCs. In contrast with enzymatic passaging were the clump size varies and the differentiated cells cannot be separated from the undifferentiated ones, with mechanical passaging the resulted cell clumps are uniform in size and it is easy to exclude the differentiated cells from the next passage. If the hESCs are needed in great amounts, might the enzymatic passaging be the best result because the enzymatic expansion produces larger amounts of hESCs in a short period of time (Oh *et al.*, 2005). However the use of enzymes and cell dissociation buffer can promote chromosomal aneuploidy, especially trisomy 12 and/or 17, and quantitative differences for gene expression (Draper *et al.*, 2004b; Mitalipova *et al.*, 2005). The karyotypic changes usually occur after extended passaging, so the enzymes may be used for shorter periods, about 15 passages, when manual dissection retains a stable karyotype even after 100 passages (Buzzard *et al.*, 2004; Mitalipova *et al.*, 2005). These two passaging techniques could be used in parallel, mechanical for maintenance of hESC lines and enzymatic for rapid expansion of cells for research or treatment purposes.

The ability of hESCs to maintain stable karyotype throughout extended culture periods is an important feature if the cells are ever to be used for transplantation, because karyotype changes *in vivo* are often associated with carcinogenesis. Not only enzymatic passaging, but other reasons as well can cause karyotypic changes, such as long-term cultivation (Inzunza *et al.*, 2004). Human ESC lines are commonly tested with traditional karyotypic analyses that are low-resolution read-outs of the genome and can miss many alterations in the DNA that may affect cellular phenotype (Maitra *et al.*,

2005). Maitra *et al.* found genomic alterations common to human cancers from late-passage hESC lines, including aberrations in copy number, mitochondrial DNA sequence and gene promoter methylation. These genetic and epigenetic alterations show that continuous analysis of hESCs during culture is important.

2.3.6. Human ESC characterization

The hESCs are characterized first hand by their morphology. Human ESCs have a high ratio of nucleus to cytoplasm, prominent nucleoli and they form colonies with sharp borders (Thomson *et al.*, 1998). A more precise characterization is done by different markers that are expressed in hESCs. Several cell-surface antigens are used to define undifferentiated hESCs, such as stage-specific embryonic antigens (SSEA-3 and SSEA-4) and tumor-related antigens (TRA-1-60 and TRA-1-81), but SSEA-1 is expressed by some of the differentiated derivatives of hESCs, notably putative trophectoderm (Henderson *et al.*, 2002; Thomson *et al.*, 1998). The undifferentiated hESCs express also high levels of alkaline phosphatase (AP) and transcription factor Oct4 (Reubinoff *et al*., 2000). Oct4 has a target gene Nanog (Chambers *et al.*, 2003), which is also used in hESC characterization. It is suggested that Nanog maintains the pluripotency of hESCs by preventing their differentiation to extra embryonic lineages (Hyslop *et al.*, 2005).

Human ESCs have a remarkable proliferation capacity, which might result from the high expression level of telomerase activity (Thomson *et al.*, 1998). In fact, human telomerase reverse transcriptase has been used to create immortalized cell line from differentiated human cells (Xu *et al.*, 2004). The telomerase activity can be used to characterize hESCs.

The important feature of hESCs is their ability to differentiate into all three embryonic germ layers. The pluripotency of stem cells must be shown before the cells can be defined as stem cells. This is done by injecting the cells into severe combined immunodeficient (SCID) mice allowing them to form teratomas, which include cells derived from all embryonic germ layers (Thomson *et al.*, 1998). An option to animal testing is to allow hESCs to aggregate in suspension and form embryoid bodies (EBs). The EBs are grown without feeder cells in suspension with medium lacking bFGF. EBs are first composed of densely packed hESCs, but later the center becomes cavitated (Itskovitz-Eldor *et al.*, 2000). These structures usually contain differentiated cells and continued culturing can result in more differentiated cells. The EBs can be grown at least up to

100 days and the differentiation can be directed using growth factors (Khoo *et al*., 2005). The different cell types can be recognized by isolating the RNA of the EBs and using PCR with primers selective for human genes or by embedding the EB on paraffin and immunoassaying the sections. Teratoma formation is still required to proof the pluripotency of new hESC lines, but EB culture should be regarded as a potential option.

2.4. Crucial issues and challenges

2.4.1. Animal-derived products

The exposure of hESCs to animal components is a matter of serious concern. There is a risk of cell rejection and contamination with retroviruses and other pathogens that could be transmitted to the patient if these cells are used in therapeutic applications. The animal components should be eliminated from all the phases of hESC culture. The derivation of hESC line is the first phase that should be optimized. Currently used methods for zona pellucida and trophectoderm removal include the use of animal derived products. Rodriguez *et al.* were the first to report the clinical-grade derivation of hESCs. They used acid Tyrore's solution instead of pronase to zona pellucida removal and the cell lines were formed without immunosurgery, thereby eliminating exposure to animal antibodies and complement factors (Rodriquez *et al*., 2006). The next step is to grow the cells without feeders, or on human feeders that are cultured without animal derived components. The culture medium must also be totally animal free. Human serum or animal-free SR must be used instead of FBS. In addition, other components should be carefully checked and chosen animal-free. Some proteins might be human recombinant but could still contain BSA as a preservative, so it is very important to check the components carefully. The passaging of the cells should be done either mechanically or with enzymes or solutions that are not animal-derived. However, avoiding animal derived products is not enough to guarantee the safety of the patient. Human feeder cells and human serum can transmit human immunodeficiency virus (HIV)-1, HIV-2, Creutzfeldt-Jakob disease, hepatitis B or C viruses and other infectious agents to the recipient (Rodriquez *et al.*, 2006). The screening of donor sources for these potential pathogens is required.

2.4.2. Good manufacturing practice

Cells used for human transplantation are regulated by the European Union. According to new EU directives (2003/94/EC and 2004/24/EC), hESCs for transplantation must be cultured using conditions resembling good manufacturing practice (GMP). Following GMP regulations record keeping, qualified personnel, high sanitary standards, cleanliness, equipment verification, validation of process and complaint management are required. The regulation enables the manufacturers to eliminate or minimize contaminations and errors, and they protect the patient from non-effective or even dangerous treatment. In order to derive clinical-grade hESC lines, the used embryos and all constituents of culture must be GMP-grade.

2.4.3. Immune response

Organ and tissue transplants between two unrelated individuals result often in graft rejection. This alloimmune response is usually controlled with immunosuppressive drugs, but it can result in numerous complications, including wound healing, infections, drug-related toxicities, skin malignancies, and low-grade lymphomas (Odorico *et al.*, 2001). Human ESC transplantation has the same immunological barriers as the conventional transplantations have.

Allelic differences between graft and host at polymorphic loci, which give rise to histocompatibility antigens, cause the rejection. Human leukocyte antigen/major histocompatibility complex (HLA/MHC) antigens are usually the most dominant immunological barriers to transplantation. The MHC class I molecules (HLA-A, HLA-B and HLA-C) and the MHC class II molecules (HLA-DR, HLA-DQ and HLA-DP) are most important (Bradley *et al.*, 2002). The HLAs of the transplant and the recipient should be similar and the HLA matching between donor and recipient is usually done by comparing three molecules, HLA-A, HLA-B and HLA-DR. Tissue allografts, which are HLA-incompatible, will induce a $CD4^+$ T-cell-dependent alloimmune response in recipients, which is more likely to result in acute graft rejection (Bradley *et al.*, 2002). But even with patients who receive a perfect matched allograft, rejection can still occur and life-long immunosuppressive therapy is needed. The MHC antigen levels in hESCs are sufficient for rejection by cytotoxic T cells, but the immunostimulatory capacity of the cells has been shown to be very low (Drukker *et al*., 2006), indicating that hESC based therapies could be a better option compared to conventional organ transplantations.

Although the immunostimulatory capacity of hESCs is low, HLA matching with the recipient would still be preferred.

The HLA matching between the hESCs and the recipient could be done by establishing stem cell banks with cell lines of many different HLA types. (Bradley *et al.*, 2002). In fact, the world's first human stem cell bank has already been established in the United Kingdom (Healy *et al.*, 2005). However, a large number of hESC lines needs to be screened before all possible haplotypes are obtained and this will take time and substantial funding.

2.4.4. Somatic-cell nuclear transfer

Perhaps the most promising method to overcome immune rejection of transplanted hESCs is somatic-cell nuclear transfer (SCNT), also known as genomic replacement and therapeutic cloning. With this technique the nucleus of an oocyte is removed and replaced by a nucleus extracted from a somatic cell of the patient. The cytoplasm of an oocyte has the ability to re-establish an embryonic gene expression program in the somatic cell nucleus, which could lead to a blastocyst development (Odorico *et al.*, 2001). This blastocyst could be used to derive a hESC line that would be custom-made for the patient (see Figure 3). Stem cells derived by SCNT should be an exact genetic match for the somatic cells of the donor, with the exception of mitochondrial genes, which are derived from the donor egg (Bradley *et al.*, 2002).

SCNT has successfully been done with animals, for example with bovine cells. Cibelli *et al.* 1998 created ES-like cells with SCNT and transplanted them to bovine embryos, which resulted in calves with at least one transgenic tissue in them (Cibelli *et al.*, 1998). The success with animals has raised the possibility of performing human SCNT for treatments of many diseases. In 2004 Hwang *et al.* reported the derivation of hESC line from cloned human embryo and later the creation of patient specific hESCs with SCNT, but these results have been revealed to be based on fabricated data (Hwang *et al*., 2005; Hwang *et al.*, 2004). So far no group has reported a successful human SCNT.

There have already been discovered many problems with the SCNT. The poor availability of human oocytes and the low efficiency of the nuclear transfer procedure reported with animals are the first obstacles to overcome. Furthermore, if hESCs could be created the long population-doubling time of the cells would make the treatment slow (Odorico *et al.*, 2001). In addition, SCNT would require the creation of a cell line

for every patient, unless the cell lines would be deposited in a stem cell bank waiting for the next patient with the same haplotype.

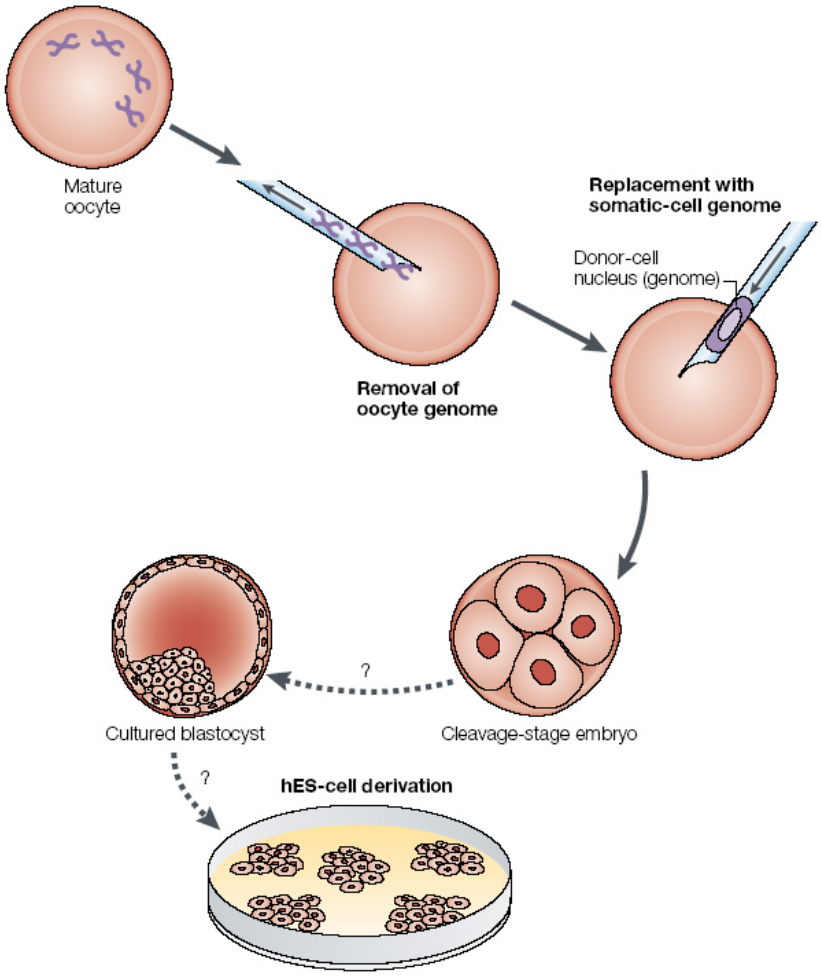


Figure 3. *Somatic-cell nuclear transfer (SCNT). The genetic material of an oocyte is removed and replaced by the genome of the patient's somatic cell by a micropipette. The oocyte cytoplasm enables the somatic-cell genome to reprogram its genes and to start embryonic development. Embryo could be cultured to blastocyst stage and the inner cell mass could be isolated. Successful human SCNT has not yet been reported, so it is unclear whether this is possible with humans. (Figure from Bradley et al. 2002).*

2.4.5. Ethical and legal issues

Although hESCs have a great therapeutic potential, they are also considered as a difficult ethical issue. Because the establishment of hESC lines involves the use of human embryos, it raises the question of ethical values at stake and the limits for such research. On the other hand, there is interest in new knowledge that can lead to treatment of incurable diseases. Opinions about the research are divided according to the different ethical, philosophical, and religious traditions.

Those who view a preimplantation embryo as a person with rights often consider the intentional destruction equivalent to murder and that human rights are violated. But does a human embryo have rights as humans do? A widely recognized definition of a person is that one must have a nervous system capable of sentience and sometimes also of cognition and consciousness. The nervous system of a human embryo starts to develop about 14 days after fertilization and the embryos used in the derivation of hESC lines are at the latest 8-day old (Stojkovic *et al.*, 2004).

The view that embryo is not a person does not mean that it is regarded as an ordinary human tissue. Some people give embryos a special respect because of their ability to develop into a human being. The use of embryos is often judged by the purpose. Creating an extended amount of embryos for IVF treatment to enable infertile couples to have children is widely accepted. The left over embryos from the treatment would be thrown away unless they were used in research. In this sense, the embryos are destroyed anyway. But the purpose of the research plays a major role in the acceptance of the hESC research. Many people accept it if the hESCs are used in developing treatments for incurable diseases but if the goal is to use them for example in cosmetic testing it seems disrespectful to the embryo. But even if the goal is to save lives some people will not accept the destroying of an embryo because no life may be taken to preserve the life of another.

Therapeutic cloning raises even more resistance in people. The creation of an embryo by SCNT is hard to accept for some people, some might even say it is like playing God. The creation of an embryo for the sole purpose of destroying it and using one part of it could objectify the embryo and further objectify also woman.

Generating hESCs by SCNT is an asexual manner, where gametes do not combine through sexual fertilization. The embryo is created for research use and later maybe to therapeutic purposes. Therapeutic cloning tends to be misrepresented in the lay media and it is often connected to reproductive cloning. However, there are no intentions to culture the embryo beyond the blastocyst stage or implant that blastocyst in a uterus for reproduction. Some fear that when therapeutic cloning is accepted it is easy to move on to reproductive cloning, but the line between these two is very clear and therefore it is not necessary to prevent therapeutic cloning in order to prevent reproductive cloning.

Laws concerning the derivation of hESC lines vary greatly throughout the world (Table 1). In Finland the medical research act of 1999 covers the preconditions and use of human embryos up to 14 days of embryonic development. The derivation of hESC

lines is also allowed. The laboratories that carry out the research need a license from the National Authority for Medicolegal Affairs and an ethics committee must give supportive statement for research projects. Also the informed consent of both gamete donors is required. In the United States, there is no federal law regulating research on human embryos or the derivation of hESC lines. However, the President of the United States, George Bush, has announced his decision to allow Federal funds to be used for research on existing hESC lines that have been derived before the announcement (2001). There are no limitations with private funding but states can have their own laws, for example, the state of California has passed a law in 2002, allowing the procurement of hESCs from supernumerary embryos. (Matthiessen-Guyader, July 2004)

Other countries, such as Norway and Ireland, forbid any research on *in vitro* embryos, stem cells and cloning, whereas Luxemburg and Portugal have no specific legislation regarding human embryo research yet (Paul *et al.*, 2002). Belgium, Sweden and United Kingdom have taken a step forward and accepted therapeutic cloning along with the derivation and research of hESCs (Matthiessen-Guyader, July 2004). Austria, Denmark, Finland, France, Germany, Greece, Ireland, Netherlands, Portugal and Spain have prohibited the creation of human embryos for research purposes in year 2003, but other countries have not yet a public position. Finland, however, has prohibited only the procurement of fertilized human embryo, which then excludes the SCNT from the law.

New regulations and a revision of the current legislation are under discussion in many countries. Stem cell research and therapeutic cloning have become a widely discussed topic in a short period of time and the legislations of several countries have not been able to keep up. All the ethical and medical aspects should be carefully considered and the resulting law should state clearly what is prohibited and what allowed.

Table 1. *Stem cell policy worldwide (Matthiessen-Guyader, July 2004)*

Countries	**Regulations**
Italy	Prohibition of the procurement of embryonic stem cells from human embryos
Austria, Spain	Prohibition of the procurement of embryonic stem cells from human embryos and the creation of human embryos for research purposes
Ireland, Norway[1]	Prohibition of the procurement of embryonic stem cells from human embryos, the research on human embryonic stem cells and the creation of human embryos for research purposes.
Germany	Prohibition of the procurement of embryonic stem cells from human embryos but allowing for the importation of the human embryonic stem cell lines derived before the year 2002. Prohibition of the creation of human embryos for research purposes.
Luxembourg, Portugal	No specific legislation regarding human embryo research. Prohibition of the creation of human embryos for research purposes.
Denmark, Finland, France, Greece, The Netherlands	Allowing for the procurement of human embryonic stem cells from supernumerary embryos. Prohibition of the creation of human embryos for research purposes.
The United States[1]	Prohibition of the procurement of embryonic stem cells from human embryos with federal fund and the research with human embryonic stem cell lines created after 2001.
Belgium, Sweden[2], United Kingdom	Allowing for the procurement of human embryonic stem cells from supernumerary embryos and the creation of human embryos for stem cell procurement.

[1] (TENK *et al*., 2003)
[2] law (2005:39) in Sweden

3. Aims of the research

There were three specific aims in this research. The first aim was to optimize culturing conditions for human embryonic stem cells (hESCs) towards animal-free conditions by testing a new totally animal-free basic fibroblast growth factor (bFGF), animal-free serum replacement that is added to the hESC culture medium and ready-made animal-free serum replacement medium. The second aim was to find an easier way for cell passaging in order to replace mechanical splicing. Animal origin free Tryple Select was tested for this purpose. The third aim was to test the possibility of growing hESCs without feeder cells using three different biomaterials.

4. Materials and Methods

4.1. Feeder cells and cell culturing plastics

Commercially available human foreskin fibroblasts (hFFs) (CRL-2429, ATCC) were used as feeder cells for human embryonic stem cells (hESCs). The hFFs were taken from liquid nitrogen, thawed and cultured using medium containing 90 % IMDM w/L-glut + Hepes (Gibco Invitrogen, USA), 10 % FBS (Gibco Invitrogen) and 50 U/ml Penicillin/Streptomycin (Cambrex, USA). The cells were cultured confluent and irradiated at 35 Gy before being plated for hESCs. Next day the medium was changed to 90 % IMDM w/L-glut + Hepes (Gibco Invitrogen), 10 % Knock-Out SR (Gibco Invitrogen) and 50 U/ml Penicillin/Streptomycin (Cambrex). The feeder plates were used up to two weeks.

Falcon 3003 tissue culture dishes and Falcon 3653 IVF cell culturing dishes (BD Biosciences, USA) were used for hFFs and hESC cultures. Also Falcon 4118 4-chamber cultureslides were used for culturing hESCs with biomaterials.

4.2. Human embryonic stem cells

With the permission of the ethics committee of the Karolinska Institute, the human embryonic stem cell (hESC) lines HS181, HS237 and HS293 have been derived from the inner cell mass (ICM) of blastocyst phase embryos, donated by couples undergoing *in vitro* fertilization (IVF) treatments in Karolinska Institute, Sweden.

The HS181 and HS237 cell lines have been originally derived and propagated using hFFs as feeder cells and using 20 % fetal bovine serum (FBS) in the hESC culture medium, but this was later changed to 20 % serum replacement (SR). HS293 cell line has not been in contact with FBS, it was derived using 20 % SR in the culture medium. HS181 and HS237 have karyotype 46,XX and HS293 has karyotype 46,XY. All the cell lines express stem cell markers specific for undifferentiated hESCs, alkaline phosphatase, Oct4, stage-specific embryonic antigen (SSEA)-4, tumor-related antigen (TRA)-1-60 and TRA-1-81 and form teratomas when injected into immunoincompetent mice. (Hovatta *et al.*, 2003; Inzunza *et al.*, 2005; Inzunza *et al.*, 2004)

4.3. Human embryonic stem cell cultures

The culture methods for hESCs used as control cells and for hESCs used in different testings are summarized in Table 2 and described more detailed below.

Table 2. *The summarized culture methods used for hESCs in this study.*

Tested method	Cell line and passages or test times	Medium	Feeders	Splitting method
Controls	HS181 (passages 49-79), HS237 (passages 60-65), HS293 (passages 41-59)	hES-medium	hFFs	Mechanical
bFGF	HS181 (tested for 16 passages), HS237 (tested for 5 passages)	hES-medium, where bFGF (R&D Systems) was replaced with bFGF from Chemicon	hFFs	Mechanical
SR-3	HS181 (tested three times) HS237 (tested two times)	hES-medium, where SR (Gibco Invitrogen) was replaced with SR-3 (Sigma-Aldrich)	hFFs	Mechanical
X-Vivo 20	HS181 (tested three times), HS237 (tested two times)	X-Vivo 20 medium (Cambrex)	hFFs	Mechanical
Tryple	HS181 (tested for 31 passages)	hES-medium	hFFs	Tryple method
Biomaterials	HS293 (tested five times)	UM hES-medium or CM hES-medium from hFFs	No feeders	No splitting

Abbreviations: hFFs, human foreskin fibroblasts; bFGF, basic fibroblast growth factor; SR, serum replacement; UM, unconditioned medium; CM, conditioned medium

4.3.1. The culture method for the hESCs used as control cells

The hESCs used as controls for the different testings were cultured using hFFs as feeder cells in hES-medium, containing 80 % Ko-DMEM (Gibco Invitrogen), 20 % Ko-SR (Gibco Invitrogen), 2mM GlutaMax (Gibco Invitrogen), 1 % Non-essential amino acids (Cambrex), 50 U/ml penicillin/streptomycin (Cambrex), 0,1 mM 2-mercaptoethanol (Gibco Invitrogen) and 8 ng/ml human recombinant basic fibroblast growth factor (hrbFGF, R & D Systems, USA). The medium was changed daily.

After 5-9 days of growing, the undifferentiated cell colonies (judged by morphology of the cells) were passaged mechanically into small pieces using a scalpel and a needle. The pieces were then transferred with a pipette onto fresh feeder plates.

The hESC lines used for testings were HS181 at passages between 49-79, HS237 at passages between 60-65 and HS293 at passages between 41-59 (one splitting equals one cell passage).

4.3.2. The culture method for hESCs used in bFGF-testing

The hrbFGF from R & D Systems used in the culture of control hESCs contains BSA and the carrier-free hrbFGF, from Chemicon, International, was tested to replace it. The culture method for hESCs in bFGF-testing was similar with the method used for control hESCs, except for the medium used that was the test bFGF medium, containing 80 % Ko-DMEM (Gibco Invitrogen), 20 % Ko-SR (Gibco Invitrogen), 2mM GlutaMax (Gibco Invitrogen), 1 % Non-essential amino acids (Cambrex), 50 U/ml penicillin/streptomycin (Cambrex), 0,1 mM 2-mercaptoethanol (Gibco Invitrogen) and 8 ng/ml or 20 ng/ml or 40 ng/ml hrbFGF (Chemicon). The hESCs from HS181 were grown in the test bFGF medium for 16 passages and HS237 for 5 passages.

4.3.3. The culture method for hESCs used in SR-3 -testing

The Ko-SR from Gibco Invitrogen used in the culture of control hESCs contains bovine serum albumine (BSA) and bovine insulin. Animal-free SR, SR-3 (Sigma-Aldrich, Germany), was tested to replace it. The hESCs used for SR-3 -testing were adapted to the SR-3 by passaging the cells onto a fresh feeder plate and using hES-medium where the amount of SR consisted the control SR (Ko-SR) and the tested SR-3 first with the ratio of 4:1. When the cells were passaged the ration was changed to 1:1, in the next passage the ratio was changed to 1:4 and finally the control SR was totally replaced by SR-3. The SR-3 was tested as 10 % and 20 % in hES-medium, where the Ko-SR was removed. The hESCs in SR-3 -testing were passaged in the same way as the control cells and the medium was changed daily. The SR-3 -testing was repeated three times with hESCs from HS181 cell line and two times with HS237 cell line.

4.3.4. The culture method for hESCs used in X-Vivo 20 -testing

Animal free SR medium, X-Vivo 20 (Cambrex), was also tested to replace the currently used animal components containing Ko-SR. The hESCs used for X-Vivo 20 -testing were adapted to the X-Vivo 20 by passaging the cells onto a fresh feeder plate and using hES-medium and X-Vivo 20 medium together first with the ratio of 4:1. When the cells were passaged the ration was changed to 1:1, in the next passage the ratio was changed to 1:4 and finally the hES-medium was totally replaced by the X-Vivo 20 -medium. The

X-Vivo 20 –medium was supplemented with 2mM GlutaMax (Gibco Invitrogen), 1 % Non-essential amino acids (Cambrex), 0,1 mM 2-mercatoethanol (Gibco Invitrogen) and 8 ng/ml hrbFGF (R&D Systems). The hESCs used in X-Vivo 20 -testing were passaged in the same way as the control cells and the medium was changed daily. The X-Vivo 20 -testing was repeated three times with hESCs from HS181 cell line and two times with HS237 cell line.

4.3.5. The culture method for hESCs used in Tryple-testing

The mechanical passaging is laborious and difficult and a new enzymatic passaging method was tested to replace it. The hESCs used for Tryple-testing were cultured in the same way as the control cells, except for the passaging method used. After 4-7 days of culture, cells were passaged using Tryple Select (Gibco Invitrogen), which is an animal-free recombinant enzyme produced with microbial fermentation. The undifferentiated colonies (judged by morphology) were lined from feeder cells by scalpel, then the hES-medium was removed and pre-warmed (+37 °C) Tryple was added to the plate (Figure 4). The cells were incubated for 1 minute and the feeder cells along with the differentiated cells were removed as a one carpet from the plate by drawing with a scalpel. The lined undifferentiated stem cell colonies stayed intact. The Tryple and the detached cells were removed from the plate with a pipette and hES-medium was added again. Then the hESC colonies could be easily spliced to smaller pieces by scalpel and removed from the bottom of the plate by scraping. The pieces were transferred to fresh feeder plates. The hESCs used for Tryple-testing were from HS181 cell line and the cells have been passaged with Tryple now for 37 times.

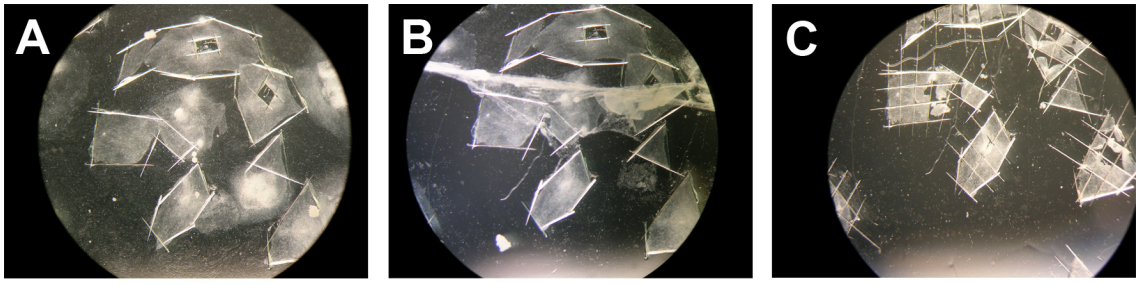


Figure 4. *The Tryple passaging method for hESCs.* ***(A)*** *The undifferentiated areas of hESC colonies were lined from the feeder cells and from the differentiated cells with a scalpel.* ***(B)*** *The hES-medium was removed and prewarmed Tryple was added to the plate. After 1 minute incubation the feeder layer and the differentiated cells were removed along with the Tryple and hES-medium was added again.* ***(C)*** *The undifferentiated areas of hESC colonies were spliced into smaller pieces by a scalpel and detached by scraping with a needle. The pieces were then transferred to fresh feeder plates.*

Different splitting techniques were tested and the method above was chosen for the actual testing. Other techniques were (1) removing the feeders around the colonies without scalpel separation, (2) collecting all the cells in one clump and pipetting it into smaller pieces or (3) scalpelling it into smaller pieces.

4.3.6. The culture method for hESCs used in biomaterial-testing

Three different biomaterials were tested for feeder-free culture of hESCs in order to get rid of the laborious production of feeder cells. Titania (TiO_2), titanium (Ti) and zirconium (Zr) coated glass pieces (10 mm x 10 mm) from Vivoxid Ltd. were placed in the chambers of a 4-chamber cultureslide. On chamber without a biomaterial was used as a control. Two different media were tested for the feeder-free culture of hESC on biomaterials, normal unconditioned hES-medium and conditioned hES-medium that was collected from hFF feeder plates after 24 h of incubation. The hESCs were passaged mechanically and the pieces were placed into the biomaterial chambers and into the chamber used as a control. The media were changed daily. The biomaterial-testing for the feeder-free culture of hESCs from HS293 cell line was repeated five times for every biomaterial.

The hESCs grown on the biomaterials were tried to passage by scraping with a needle, but the cells detached as single cells and did not attach to the biomaterials anymore. The hESC colonies grown on the biomaterials were detached gently by a tip of a pipette in as big pieces as possible and transferred back on feeder cells. The cell pieces attached to the feeder cells and were allowed to proliferate.

4.3.7. Statistics and calculations

The numbers of undifferentiated, partly differentiated and differentiated hESC colonies were counted before every passage in all culture conditions and the percentage values were calculated, in order to see how the test conditions maintain and support the expanded growth of hESCs. The better the condition maintain the undifferentiated growth of hESCs, the more new plates can be obtained from one culture plate after passaging, expanding the growth of hESCs.

The areas of the undifferentiated hESC colonies were measured daily, using the EclipseNet computer program (Nikon, USA). The areas of the colonies could be measured only from the hESC colonies grown with control, test bFGF and Tryple culture methods, because there were no undifferentiated colonies in SR-3 and X-Vivo 20

testings after the adaptation of the cells and the hESCs grown on the biomaterials had entirely different morphology than the control cells, making the determination of the undifferentiated cells impossible. The measured areas were used to calculate the instant growth rates (G values) of the colonies. The G-values were calculated using the following formula: $G=\ln[(A_2/A_1)/\Delta t]$, where A_1 is the area of the colony at time point 1, A_2 is the area of the colony at time point 2 and Δt is the change of time. The data is expressed as mean ± SD. The differences between groups was analyzed using one-way ANOVA and the differences were considered statistically significant at $p < 0,05$.

4.4. Embryoid body formation

The pluripotency of the hESCs cultered with Tryple-method was showed by culturing embryoid bodies (EBs). The EBs were formed by mechanically dissecting undifferentiated hESC colonies and transferring the resulted pieces onto a culture dish without feeder cells. The EBs were cultured in hES-medium without bFGF approximately for 30 days before the isolation of RNA and approximately for 70 days before immunoassaying. The medium was changed every 2-3 days.

4.5. Immunochemistry

4.5.1. Immunofluorescense analysis of hESCs

The hESCs grown with the bFGF, SR-3, X-Vivo 20 and Tryple culture method were determined undifferentiated or differentiated by immunocytochemistry. Also the hESC colonies grown on feeder cells after one passage on the biomaterials were analyzed in the same way. The primary antibodies used against markers expressed in undifferentiated hESCs were goat anti-Nanog and goat anti-Oct4, both at 1:100 dilutions. Primary antibody used against differentiated hESC marker was mouse anti-SSEA-1 (1:100). All the primary antibodies were from Human Embryonic Stem Cell Marker Antibody Panel (SC008, R&D Systems). Secondary antibodies used were Alexa Fluor 488 donkey anti-goat IgG (1:600) (Molecular Probes Invitrogen, USA) for anti-Nanog and anti-Oct4 and Rhodamine Red conjugated donkey anti-mouse IgM (1:800) (Jackson Immunoresearch Laboratories, USA) for anti-SSEA-1. All experiments included negative controls for staining where fibroblasts or only the secondary antibody were used.

The hESCs were fixed with 4 % paraformaldehyde for 20 minutes at room temperature and washed 3 times for 5 minutes in phosphate buffered saline (PBS, Cam-

brex). The immunostaining was performed according to the following protocol: (1) the cells were permeabilized and the non-specific binding of the antibody was blocked with treatment in 0.1 % Triton X-100 (Sigma-Aldrich), 1 % BSA (Sigma-Aldrich), 10 % normal donkey serum (Sigma-Aldrich) in PBS at room temperature for 45 minutes; (2) the cells were incubated with primary antibodies diluted in 1 % BSA, 1 % normal donkey serum in PBS overnight at +4 °C. For negative control only the dilution solution was used. The cells were then washed 3 times for 5 minutes in PBS containing 1 % BSA; (3) the cells were incubated with secondary antibodies diluted in PBS containing 1 % BSA for 60 minutes at room temperature in the dark. The cells were then washed 2 times for 5 minutes in PBS and 2 times for 5 minutes in phosphate buffer (PB).

Finally, the cells were mounted in Vectashield with DAPI (Vector Laboratories, USA) and covered with a cover-slip. The cells were examined with a Nikon Eclipse TE2000-S microscope and photographed with a Nikon Coolpix 5400 camera.

4.5.2. Immunoperoxidase staining of hESCs

Undifferentiated hESCs express high levels of Alkaline Phosphatase (AP). The undifferentiated state of hESCs grown with the Tryple and with the bFGF culture method was analyzed using the Alkaline Phosphatase Detection Kit (Chemicon).

The hESCs were fixed with 4 % paraformaldehyde for 2 minutes at room temperature and washed 3 times for 5 minutes in phosphate buffered saline (PBS, Cambrex). The AP staining was performed according to the following protocol: (1) Fast Red Violet (FRV, supplied with the kit), Naphthol AS-BI phosphate solution (supplied with the kit) and water were mixed in a 2:1:1 ratio. The hESCs were incubated in the stain solution for 15 minutes at room temperature in the dark; (2) the cells were then washed 3 times for 5 minutes in PBS and examined with a Nikon Eclipse TE2000-S microscope and photographed with a Nikon Coolpix 5400 camera. The hESCs expressing AP were stained red and the differentiated hESCs were colorless.

4.5.3. Immunofluorescense analysis of the sections of EBs

The pluripotency of the hESCs grown with the Tryple culture method was shown by analyzing the sections of EBs by immunohistochemistry. The EBs were embedded in paraffin and 3 μm sections were cut and placed on microscope slides by Histola Ltd. The primary antibodies used against ectodermal markers were goat anti-Sox-1 (1:20, Santa Cruz) and mouse anti-nestin (1:100, Chemicon), against endodermal marker was

goat anti-alphafetoprotein (AFP 1:20, Santa Cruz, USA), and against mesodermal marker was mouse anti α-sarcomeric actin (α-sr-1, 1:100, Sigma-Aldrich). Secondary antibodies (all from Jackson Immunoresearch Laboratories) used were Rhodamine Red conjugated donkey anti-goat IgG for anti-Sox-1 and anti-AFP, Rhodamine Red conjugated donkey anti-mouse IgG for anti-Nestin and Rhodamine Red conjugated donkey anti-mouse IgM for anti α-sarcomeric actin. All experiments included negative controls for staining where fibroblasts or only the secondary antibody were used.

The EB samples on the microscope slides were deparafinized by incubating them in the following solutions: 2 times for 5 minutes in xylene (Sigma-Aldrich), 2 times for 5 minutes in absolute ethanol, for 5 minutes in 95 % ethanol, for 3 minutes in 90 % ethanol, for 3 minutes in 85 % ethanol and 3 times for 1 minute in distilled water. The EB samples on the slides were then treated with antigen retrieval citrate buffer for 20 minutes at +95 °C, followed by 20 minute cooling at room temperature. The EB samples on the slides were washed 2 times for 2 minutes in distilled water.

The immunostaining was performed according the protocol described in section 4.7.1 and finally, the EB samples were mounted in Vectashield with DAPI (Vector Laboratories) and covered with a cover-slip. The EB samples were examined with a Nikon Eclipse TE2000-S microscope and photographed with a Nikon Coolpix 5400 camera.

4.6. RNA extraction and reverse transcription

Total RNA was isolated from EBs differentiated from the hESCs grown with the Tryple culture method by using RNeasy micro kit (Qiaken, Germany). The RNA extraction was performed according to the following protocol: (1) 350 µl of Buffer RLT (supplied with the kit) with added β-Mercaptoethanol (Sigma-Aldrich) was added per two or three embryoid bodies, and the sample was homogenized by vortexing; (2) 350 µl of 70 % ethanol was added, and the suspension was mixed by pipetting. The sample was applied to an RNeasy MinElute Spin Column in a 2 ml collection tube (supplied with the kit) and centrifuged for 15 s at 10,000 rpm. The flow-through was discarded; (3) 350 µl Buffer RW1 was added to the column and centrifuged for 15 s at 10,000 rpm to wash the column. The flow-through was discarded; (4) 10 µl of DNase I stock solution (supplied with the kit) was added to 70 µl of Buffer RDD (supplied with the kit) and mixed by inverting the tube. The DNase I incubation mix (80 µl) was pipetted onto the silica-

gel membrane of the column and incubated for 15 minutes at room temperature; (5) 350 µl of Buffer RW1 was added to the column and centrifuged for 15 s at 10,000 rpm. The flow-through and the collection tube were discarded; (6) the column was transferred into a new collection tube, 500 µl of Buffer RPE (supplied with the kit) was added to the column and centrifuged for 15 s at 10,000 rpm to wash the column. The flow-through was discarded; (7) 500 µl of 80 % ethanol was added to the column and centrifuged for 2 minutes at 10,000 rpm to dry the silica-gel membrane. The flow-through and the collection tube were discarded; (8) The column was transferred into a new 2 ml collection tube. The cap of the spin column was opened and centrifuged for 5 minutes at 15,000 rpm. The flow-through and the collection tube were discarded; (9) for eluation, the column was transferred to a new 1,5 ml collection tube. 14 µl of RNase-free water was pipetted onto the center of the silica-gel membrane and centrifuged for 1 minute at 15,000 rpm. The concentration and quality of isolated RNA was studied by measuring absorbance at 260 and 280 nm with a ND-1000 Spectrophotometer (NanoDrop Technologies, USA).

Complementary DNA (cDNA) was synthesized from 50 ng of total RNA using Sensiscript Reverse Transcription Kit (Qiaken). 2 µl of Oligo-dT primer (10 µM, Fermentas, USA) and 1 µl RNase inhibitor (40 units/µl, Fermentas) diluted in 1x Buffer RT for a concentration of 10 units/µl were mixed with 50 ng of template RNA. 2 µl of 10x Buffer RT, 2 µl of dNTP Mix (5 mM each dNTP) and 1 µl of Sensiscirpt Reverse Transcriptase (all supplied with the kit) were added to the reaction mixture. RNase-free water was added so that the final volume of the mixture was 20 µl. The reaction mixture was incubated for 60 minutes at +37 °C.

4.7. RT-PCR and agarose gel electrophoresis

In addition to the immunochemistry analysis of EB sections, the pluripotency of the hESCs grown with the Tryple culture method was shown also by performing reverse transcription-PCR (RT-PCR) using cDNA, made from the extracted RNA of the EBs, as a template. Primers, summarized in Table 3, were used to detect the expression of markers characteristic of ectoderm (neuroD-1, neurofilament 68KD), endoderm (α-fetoprotein) and mesoderm (α-cardiac actin). Glyseraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping control.

Approximately 500 ng of cDNA was used as template in the PCR reactions. Negative control contained sterilized water instead of cDNA template. All the reagents used were from Fermentas. The PCR reaction was as follows: 17.15 µl sterilized water, 2.5 µl 10x Taq buffer with KCl, 0.2 µl 25 mM dNTP mix, 1 µl of 5 µM primer-F, 1 µl of 5 µM primer-R, 2 µl 25 mM $MgCl_2$, 0.15 µl Taq DNA Polymerase and 1 µl cDNA. The PCR reactions were carried out in the Eppendorf Mastercycler as follows: denaturation at 95 °C for 3 minutes and 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 minute, followed by final extension at 72 °C for 5 minutes.

The PCR products were analyzed by electrophoresis on 1.5 % agarose gel containing 0.1 µg/ml ethidium bromide (Sigma-Aldrich) and DNA standard (MassRuler™ DNA Ladder Mix, Fermentas).

Table 3. *PCR primers (Proligo, USA) used to detect gene expression in EBs.*

Gene product	Forward (F) and reverse (R) primers (5'-3')	Product size (bp)
ND-1	F: AAGCCATGAACGCAGAGGAGGACT	579
	R: AGCTGTCCATGGTACCGTAA	
NF-68KD	F: GAGTGAAATGGCACGATACCTA	473
	R: TTTCCTCTCCTTCTTCACCTTC	
AFP	F: GCTGGATTGTCTGCAGGATGGGGAA	216
	R: TCCCCTGAAGAAAATTGGTTAAAAT	
α-cardiac actin	F: GGAGTTATGGTGGGTATGGGTC	486
	R: AGTGGTGACAAAGGAGTAGCCA	
GAPDH	F: AGCCACATCGCTCAGACACC	302
	R: GTACTCAGCGGCCAGCATCG	

Abbreviations: ND-1, neuroD-1; NF-68KD, neurofilament 68 KD; AFP, α-fetoprotein; GAPDH, glyseraldehyde 3-phosphate dehydrogenase; bp, base pairs

4.8. Karyotyping

Because long-term enzymatic passaging method has been shown to cause karyotypic changes in hESCs, especially trisomy 12 and 17, the karyotype of hESCs passaged with the enzyme Tryple was determined. The karyotype analysis of hESCs grown with the Tryple culture method for 31 passages was performed by Laboratoriokeskus Ltd Tampere, Finland.

Approximately 25 individual cells at mitosis were analyzed by Giemsa-banding (G-banding) and further testing of the cells was done by fluorescence *in situ* hybridization (FISH) analysis of chromosomes chosen based on the result of the G-banding.

5. Results

5.1. The results of the test bFGF culture method for hESCs

The human recombinant basic fibroblast growth factor (hrbFGF, from R & D Systems) used in hES-medium for hESCs used as control cells contains bovine serum albumine (BSA), and new hrbFGF from Chemicon without BSA was tested to replace it. The morphology of the hESC colonies in test bFGF culture method was thinner compared to control colonies with both cell lines (HS181 and HS237) used for testing (Figure 5), which made the test cells more difficult to passage mechanically.

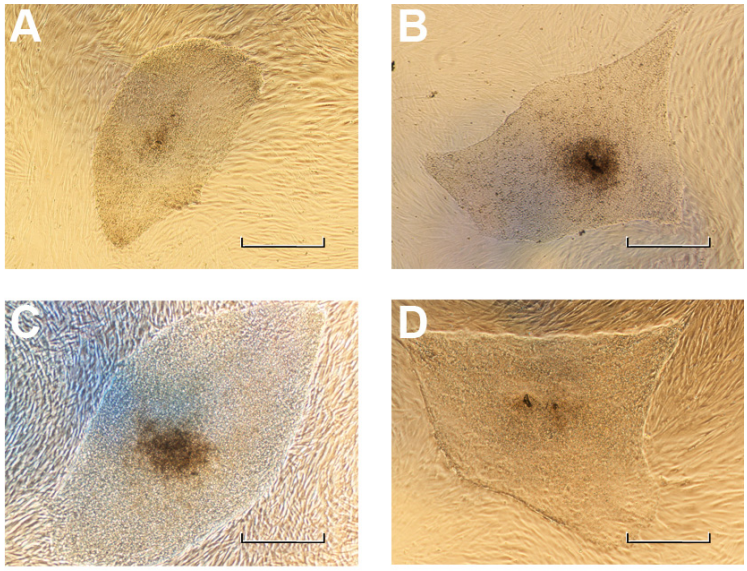


Figure 5. *The morphology of the hESC colonies grown with the control culture method and with the test bFGF culture method.* ***(A)*** *HS181 control cells at passage 67, five days after splitting;* ***(B)*** *HS181 cells grown for 1 passage with 8 ng/ml of test bFGF, four days after passaging;* ***(C)*** *HS237 control cells at passage 63, six days after passaging;* ***(D)*** *HS237 cells grown for 3 passages with 8 ng/ml of test bFGF, five days after passaging. Magnification 4x. Scale bar 500 µm.*

The level of differentiation was judged by the morphologies of the colonies before they were passaged. With HS237 cells, the control bFGF promoted the growth of undifferentiated cells better and there were fewer differentiated colonies compared to any concentration (8, 20 and 40 ng/ml) of the test bFGF (Figure 6). Similar results were obtained with HS181 cell line (data not shown). The test bFGF seemed to maintain undifferentiated hESC growth better as the concentration was raised, but it did not reach the control with the concentrations tested.

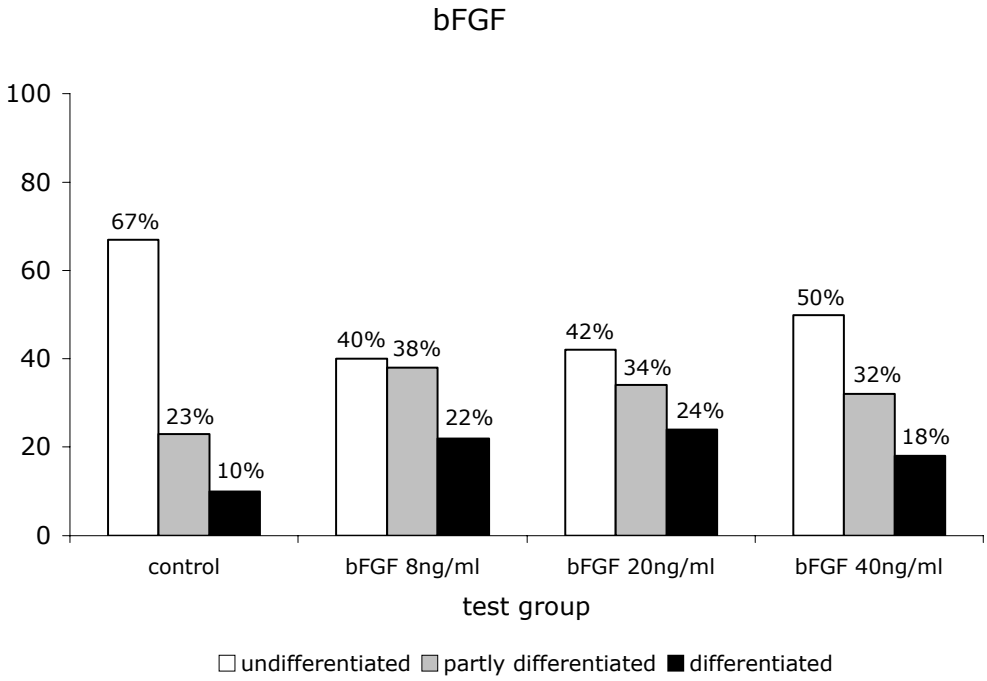


Figure 6. *The level of differentiation of hESC colonies grown with the test bFGF culture method using 8, 20 and 40 ng/ml of test bFGF compared to the hESC colonies grown with the control culture method. n=198 for control group, n=53 for test bFGF 8 ng/ml group, n=65 for test bFGF 20 ng/ml group and n=28 for test bFGF 40 ng/ml group. n=number of colonies.*

The areas of the undifferentiated hESC colonies were measured daily and four different instant growth rates (G values) were calculated to each colony, between days 2-3 (G1), 3-4 (G2), 4-5 (G3) and 5-6 after passaging (G4). The mean values of the test groups were compared to the mean value of the control group by one-way ANOVA for each time point (Figure 7). The differences between the groups were not statistically significant at $p > 0,05$, but the G values of hESC colonies grown with test bFGF culture method were the same or slightly higher than the G values of the control colonies almost every time.

The undifferentiated hESC colonies (judged by the morphology) grown with the test bFGF in all tested concentrations expressed markers common to undifferentiated hESCs (Nanog, Oct4, Alkaline phosphatase) and were negative to marker common to differentiated hESCs (SSEA-1) (Figure 8 and 9).

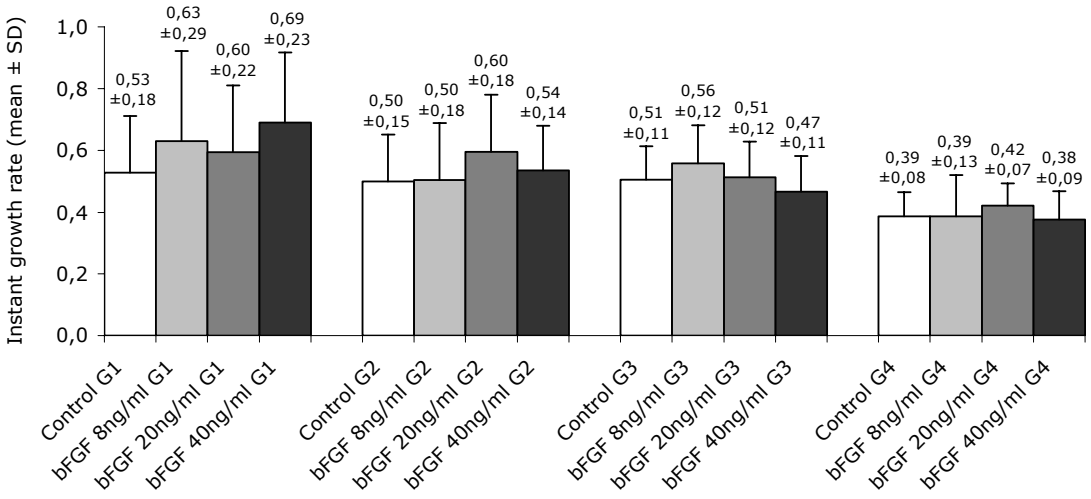


Figure 7. *The instant growth rates of undifferentiated hESC colonies grown with the test bFGF culture method compared to the colonies grown with the control culture method. The instant growth rates of the colonies in four different time points (between days 2-3, G1; days 3-4, G2; days 4-5, G3 and days 5-6 after passaging, G4) are presented as mean values.*

Regardless of the similar instant growth rates of the hESC colonies grown with the test bFGF culture method and the culture method used for control cells, the test bFGF culture method did not expand the growth of hESCs equally well as the control culture method, because of the low percentage value of the undifferentiated colonies with the test bFGF culture method. When similar amount of pieces of hESC colonies are transferred to new plate in passaging, the undifferentiated hESCs from one culture plate grown with the test bFGF culture method could be passaged on to only one new culture plate, where as hESCs from one culture plate grown with the control culture method could usually be passaged on to three new culture plates, expanding the growth of hESCs (data not shown).

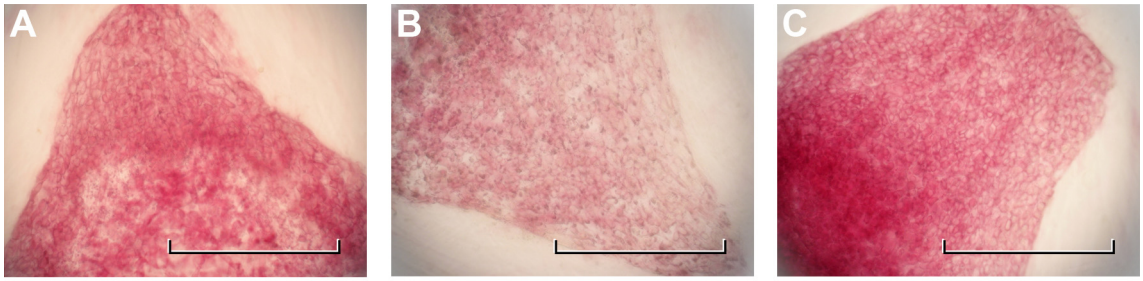


Figure 8. *The expression of Alkaline Phosphatase in the undifferentiated hESC colonies grown with the test bFGF culture method.* ***(A)*** *hESC colony grown for 5 passages with 8 ng/ml of test bFGF;* ***(B)*** *hESC colony grown for 5 passages with 20 ng/ml of test bFGF;* ***(C)*** *hESC colony grown for 5 passages with 40 ng/ml of test bFGF. Magnification 10x, scale bar 500 µm.*

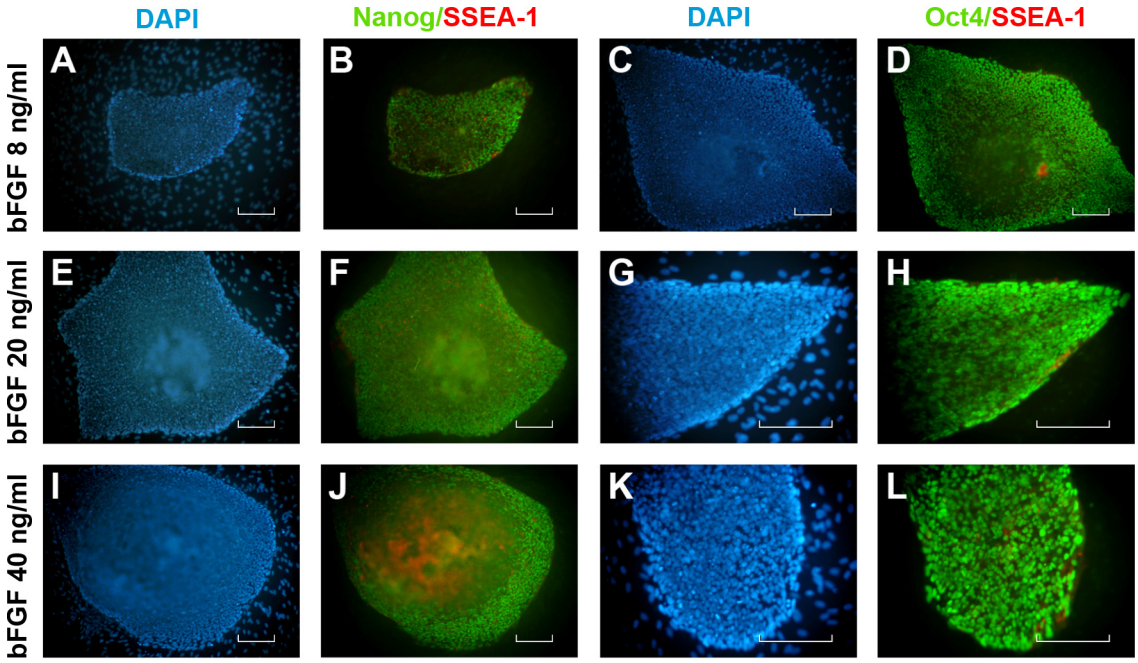


Figure 9. *The expression of markers common to undifferentiated hESCs (Nanog and Oct4) and a marker common to differentiated hESCs (SSEA-1) in hESC colonies grown with the test bFGF culture method.* ***(A)*** *hESC colony grown for 5 passages with 8 ng/ml of test bFGF, five days after passaging, stained with DAPI and* ***(B)*** *showing the expression of Nanog(green) and SSEA-1(red), magnification 10x;* ***(C)*** *hESC colony grown for 5 passages with 8 ng/ml of test bFGF, five days after passaging, stained with DAPI and* ***(D)*** *showing the expression of Oct4(green) and SSEA-1(red), magnification 10x;* ***(E)*** *hESC colony grown for 5 passages with 20 ng/ml of test bFGF, five days after passaging, stained with DAPI and* ***(F)*** *showing the expression of Nanog(green) and SSEA-1(red), magnification 10x;* ***(G)*** *hESC colony grown for 5 passages with 20 ng/ml of test bFGF, five days after passaging, stained with DAPI and* ***(H)*** *showing the expression of Oct4(green) and SSEA-1(red), magnification 20x;* ***(I)*** *hESC colony grown for 5 passages with 40 ng/ml of test bFGF, five days after passaging, stained with DAPI and* ***(J)*** *showing the expression of Nanog(green) and SSEA-1(red), magnification 10x;* ***(K)*** *hESC colony grown for 5 passages with 40 ng/ml of test bFGF, five days after passaging, stained with DAPI and* ***(L)*** *showing the expression of Oct4(green) and SSEA-1(red), magnification 20x. Scale bar 200 µm.*

5.2. The results of the SR-3 culture method for hESCs

The serum replacement, SR-3 from Sigma-Aldrich, was tested on hESC culture by replacing the currently used SR (Gibco Invitrogen) that contains animal-derived components. The presence of SR-3 in hESC culture medium did not promote the maintenance of undifferentiated cells. The results were consistent in both hESC lines examined (HS237 and HS181). The data presented here is from HS237 cells. The number of undifferentiated colonies diminished significantly after second adaptation phase (1:1, SR-3:control SR) with both SR-3 concentrations tested (10 % and 20 %) and was lost completely when the control SR was left aside (Figure 10 and 11). The differentiation

was first judged by morphology and then confirmed by immunocytochemistry. The hESC colonies grown with the SR-3 culture method in both tested concentrations expressed a marker common to differentiated hESCs (SSEA-1) and were negative to a marker common to undifferentiated hESCs (Nanog) (Figure 13).

5.3. The results of the X-Vivo 20 culture method for hESCs

The tested animal-free X-Vivo 20 serum replacement medium did not maintain the growth of undifferentiated cells from hESC lines HS181 and HS237. The data presented here is from HS237 cells. The number of differentiated colonies grew as the amount of X-Vivo 20 medium was increased and the amount of hES-medium decreased. When there was no hES-medium present, all the colonies were differentiated (Figure 12). The differentiation was first judged by morphology and then confirmed by immunocytochemistry. The hESC colonies grown with the X-Vivo 20 culture method expressed a marker common to differentiated hESCs (SSEA-1) and were negative to a marker common to undifferentiated hESCs (Nanog) (Figure 13).

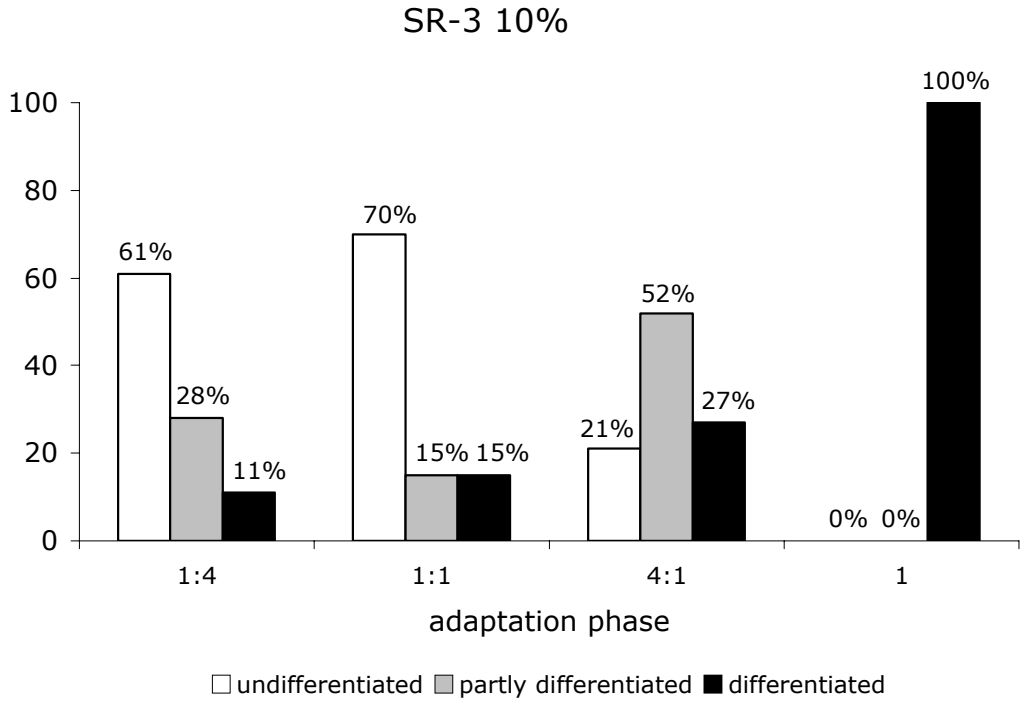


Figure 10. *The level of differentiation of hESC colonies grown with the SR-3 culture method in the adaptation phases and after the adaptation using 10 % SR-3. n=18 for adaptation phase 4:1 (Ko-SR:SR-3), n=33 for adaptation phase 1:1, n=62 for adaptation phase 1:4 and n=12 after the adaptation. n=number of colonies.*

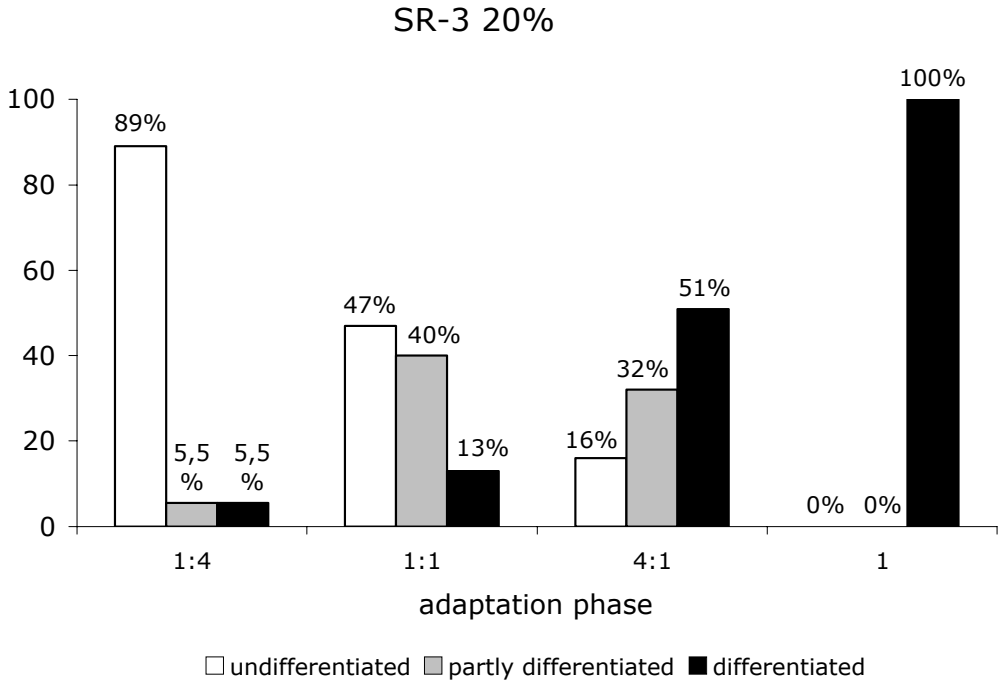


Figure 11. *The level of differentiation of hESC colonies grown with the SR-3 culture method in the adaptation phases and after the adaptation using 20 % SR-3. n=18 for adaptation phase 4:1 (Ko-SR:SR-3), n=15 for adaptation phase 1:1, n=37 for adaptation phase 1:4 and n=7 after the adaptation. n=number of colonies.*

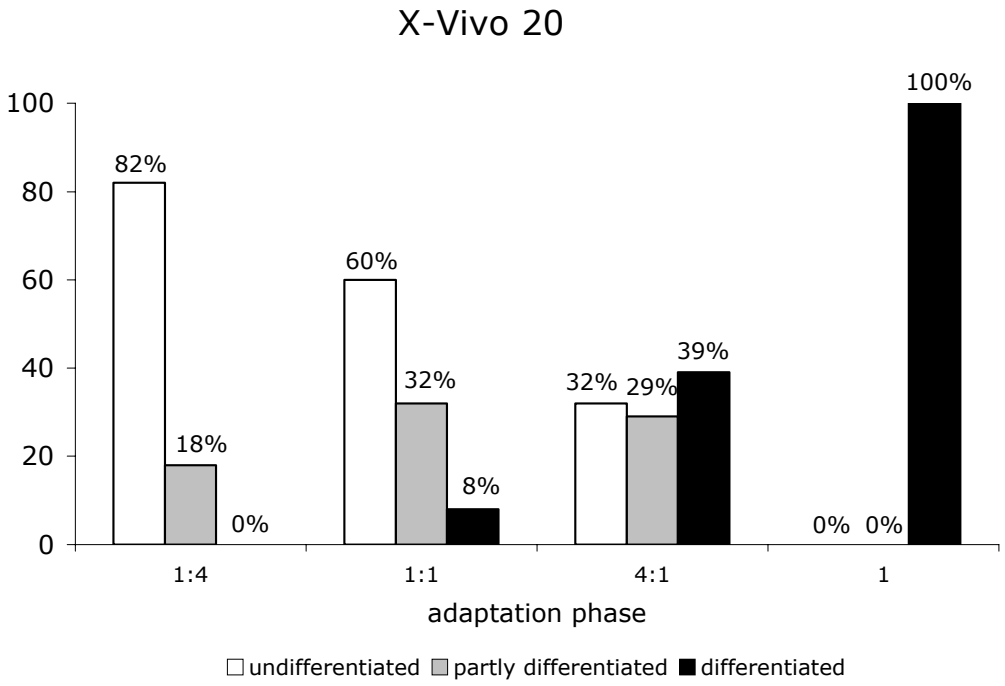


Figure 12. *The level of differentiation of hESC colonies grown with the X-Vivo 20 culture method in the adaptation phases and after the adaptation. n=17 for adaptation phase 4:1 (hES-medium:X-Vivo 20 medium), n=25 for adaptation phase 1:1, n=28 for adaptation phase 1:4 and n=9 after the adaptation. n=number of colonies.*

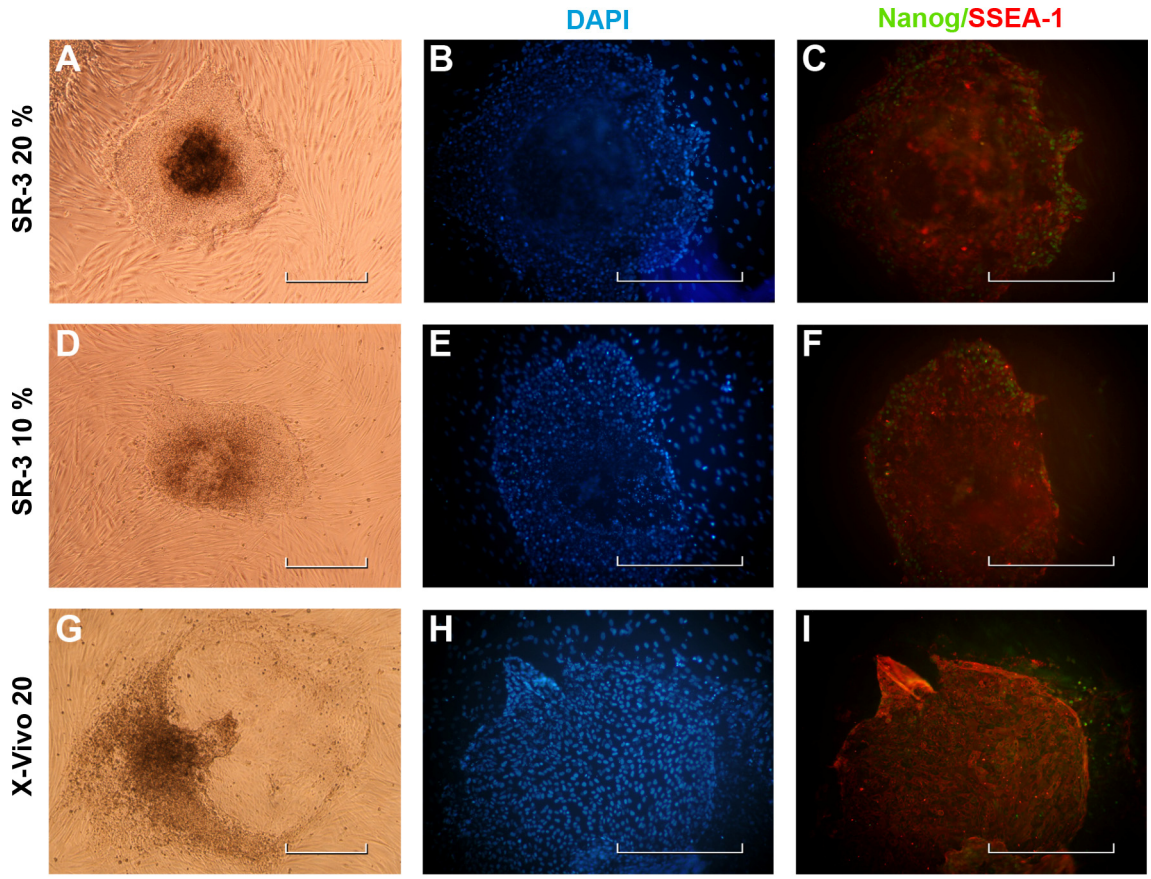


Figure 13. *The expression of a marker common to undifferentiated hESCs (Nanog) and a marker common to differentiated hESCs (SSEA-1) in hESC colonies grown with the SR-3 culture method with both tested concentrations of SR-3 (10 % and 20 %) and in hESC colonies grown with the X-Vivo 20 culture method.* ***(A)*** *hESC colony grown with the SR-3 culture method using 10 % SR-3, magnification 4x,* ***(B)*** *stained with DAPI and* ***(C)*** *showing the expression of Nanog (green) and SSEA-1 (red), magnification 10x;* ***(D)*** *hESC colony grown with the SR-3 culture method using 20 % SR-3, magnification 4x,* ***(E)*** *stained with DAPI and* ***(F)*** *showing the expression of Nanog (green) and SSEA-1 (red), magnification 10x;* ***(G)*** *hESC colony grown with the X-Vivo 20 culture method, magnification 4x,* ***(H)*** *stained with DAPI and* ***(I)*** *showing the expression of Nanog (green) and SSEA-1 (red), magnification 10x. Scale bar 500 μm.*

5.4. The results of the Tryple culture method for hESCs

The animal-free enzyme Tryple was preliminary tested for the passaging of hESCs by four different ways and one was chosen for the Tryple culture method (Figure 4 on page 36). The other three techniques were (1) removing the feeders around the colonies without scalpel separation, (2) collecting all the cells in one clump and pipetting it into smaller pieces or (3) scalpelling it into smaller pieces. These other three techniques failed, mainly because of the tight cell-cell interactions of hESC colonies. If the colonies were not lined with a scalpel, they detached with feeder cells and the effort to make

smaller pieces from one big cell clump by pipetting was not successful. The splitting could be done with a scalpel, but it was difficult and the resulted pieces were not small enough.

The levels of differentiation of hESC colonies grown with the Tryple culture method were different from those grown with the control culture method. There were fewer partly differentiated colonies grown with the Tryple culture method than with the control culture method but the amount of undifferentiated colonies was higher (Figure 14). The extended growth of hESCs grown with the Tryple culture method was more efficient than with the control culture method, because of the higher amount of undifferentiated colonies with the Tryple culture method and because with the Tryple passaging method the hESC colonies could be passaged into even smaller pieces than the colonies grown with the control culture method (data not shown). This could be done because the pieces of hESC colonies were easier to detach from the plate after the Tryple treatment and it did not demand accurate needle handling.

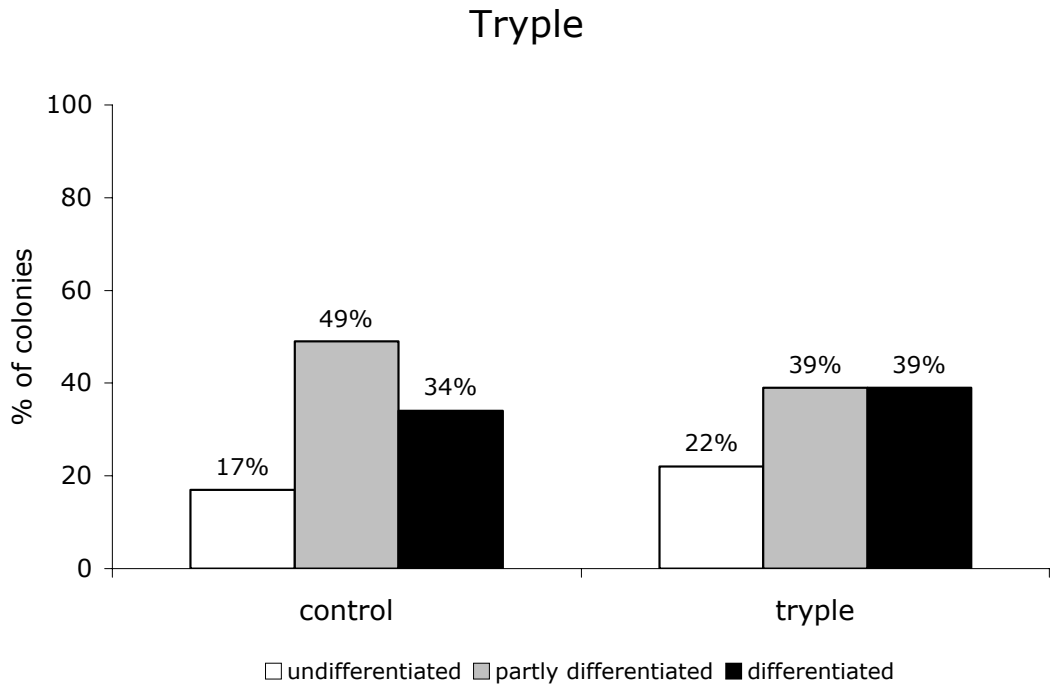


Figure 14. *The level of differentiation of hESC colonies grown with the Tryple culture method compared to the colonies grown with the control culture method. n=723 for the control group and n=1109 for the Tryple group. n=number of colonies.*

The areas of the undifferentiated hESC colonies were measured daily and three different instant growth rates (G values) were calculated to each colony, between days 2-3 (G1), 3-4 (G2) and 4-5 (G3) after passaging. The area of the colonies could not be

measured after day 5, because HS181 cells grow faster than HS237 cells and the colonies did not fit into the picture taken by Nikon EclipseNet anymore. The mean values of the Tryple groups were compared to the mean values of the control group by one-way ANOVA for each time point (Figure 15). The differences between the groups were not statistically significant at $p > 0,05$.

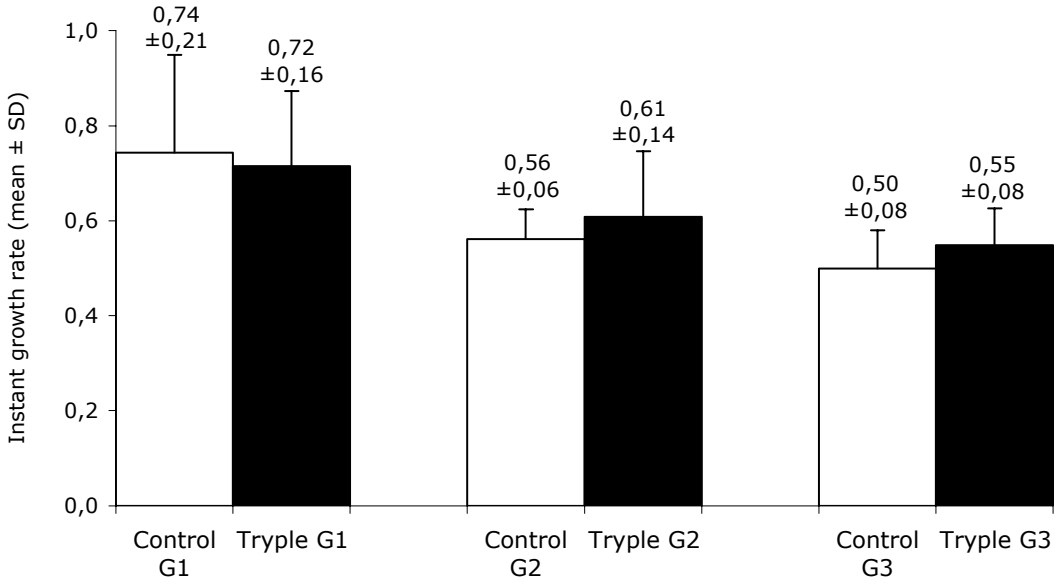


Figure 15. *The instant growth rates of undifferentiated hESC colonies grown with the Tryple culture method compared to the colonies grown with the control culture method. The instant growth rates of the colonies in three different time points (between days 2-3, G1; days 3-4, G2 and days 4-5 after passaging, G3) are presented as mean values.*

The undifferentiated hESC colonies (judged by the morphology) grown with the Tryple culture method expressed markers common to undifferentiated hESCs (Nanog, Oct4, Alkaline phosphatase) and were negative to marker common to differentiated hESCs (SSEA-1) (Figure 16 and 17).

The EBs were differentiated from hESCs to demonstrate the pluripotency of hESCs grown with the Tryple culture method. cDNA was made from the extracted RNA of the EBs and used in the RT-PCR analysis, which showed the differentiation of hESCs towards all the embryonic germ layers: ectoderm (ND-1 and NF-68), mesoderm (α-cardiac actin) and endoderm (AFP) (Figure 18). Although RT-PCR is only a semi quantitative method when similar amount of cDNA is used in the reactions, it could be said that these hESCs preferred to differentiate towards the ectoderm.

The pluripotency of the cells was also confirmed at the protein expression level by immunofluorescense analysis of the sections of EBs using markers specific for the embryonic germ layers (ectoderm: sox-1 and Nestin, mesoderm: α-sarcomeric actin and endoderm: AFP) (Figure 19).

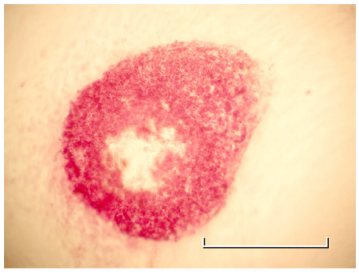


Figure 16. *The expression of Alkaline Phosphatase (common to undifferentiated hESCs) in hESC colony grown for 6 passages with the Tryple culture method, five days after passaging. Magnification 10x. Scale bar 500 μm.*

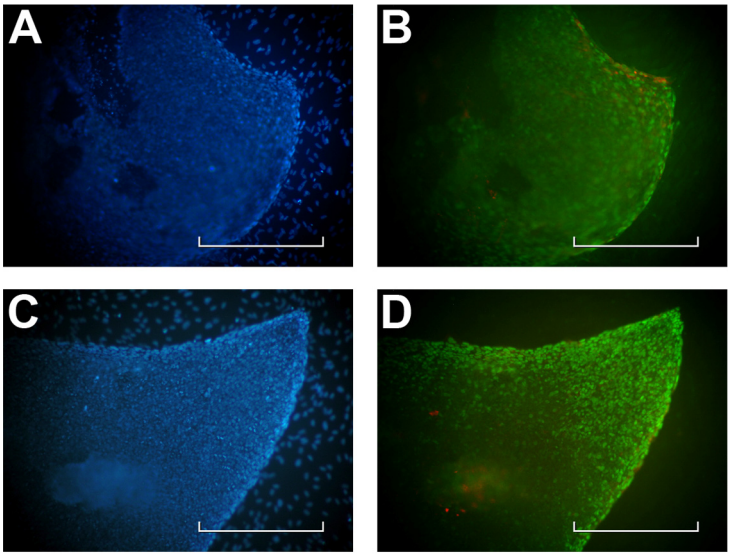


Figure 17. *The expression of markers common to undifferentiated hESCs (Nanog and Oct4) and a marker common to differentiated hESCs (SSEA-1) in hESC colonies grown with the Tryple culture method.* ***(A)*** *hESC colony grown for 5 passages with the Tryple culture method, six days after passaging, stained with DAPI and* ***(B)*** *showing the expression of Nanog (green) and SSEA-1 (red), magnification 10x;* ***(C)*** *hESC colony grown for 6 passages with the Tryple culture method, five days after passaging, stained with DAPI and* ***(D)*** *showing the expression of Oct4 (green) and SSEA-1 (red), magnification 10x. Scale bar 500 μm.*

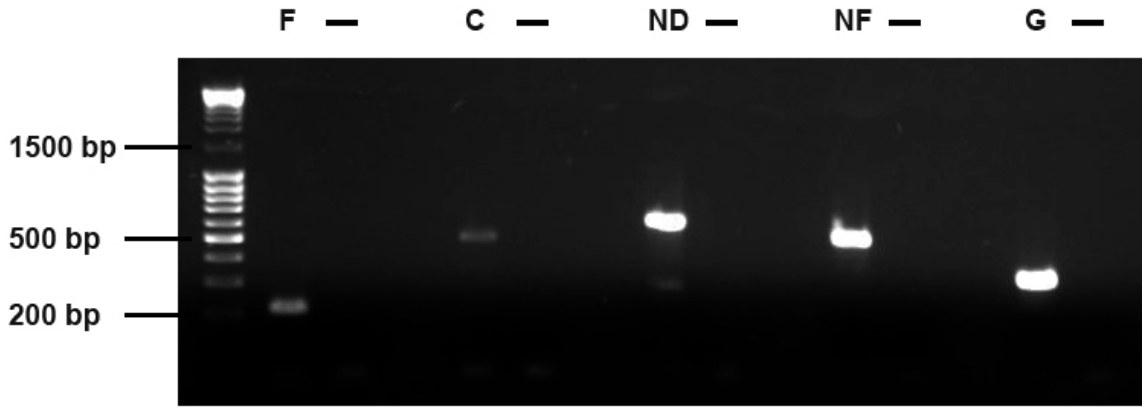


Figure 18. *RT-PCR analysis of embryoid bodies (EBs) differentiated from the hESCs grown with the Tryple culture method. EBs expressed genes specific for* ***endodermal layer****, α-fetoprotein (F, 216 bp);* ***mesodermal layer****, α-cardiac actin (C, 486 bp) and* ***ectodermal layer****, ND-1 (ND, 579 bp) and NF-68 (NF, 473 bp). GAPDH (G, 302 bp) was used as a housekeeping control and water control (-) as negative control for each primer pair.*

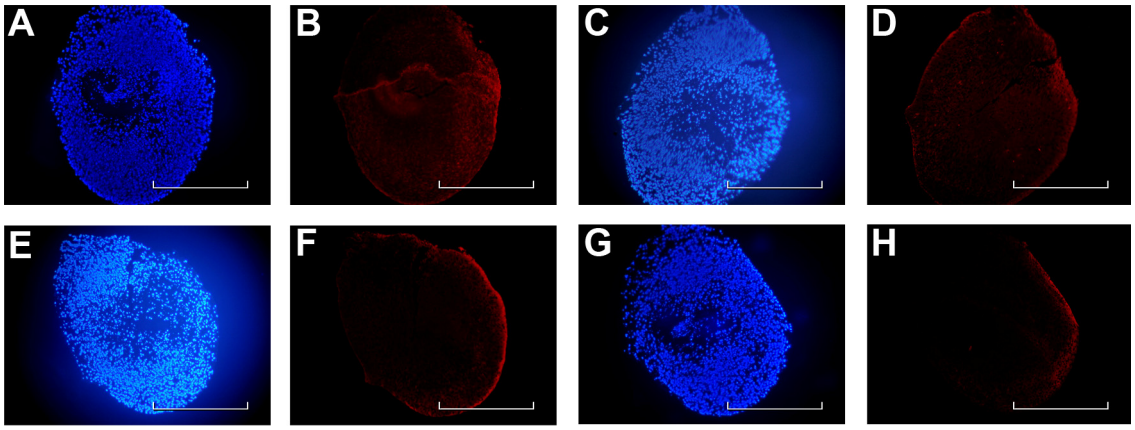
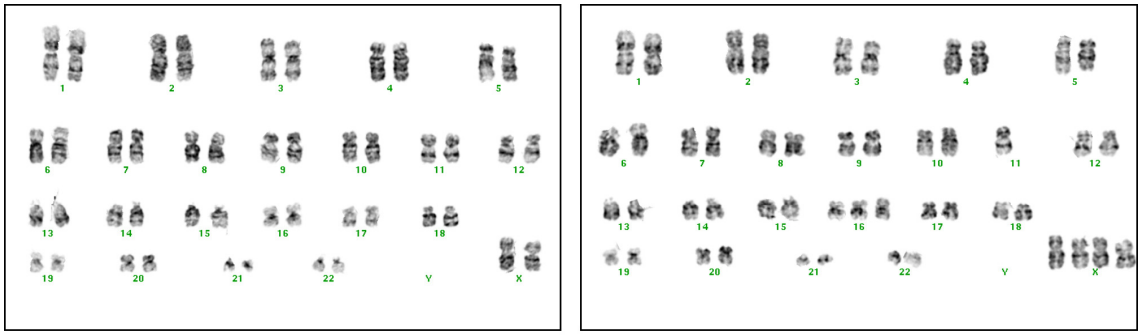


Figure 19. *The immunofluorescense analysis of the sections of EBs differentiated from the hESCs grown for 16 passages with the Tryple culture method.* ***(A)*** *A section of EB stained with DAPI and* ***(B)*** *showing the expression of Sox-1 (ectodermal marker);* ***(C)*** *A section of EB stained with DAPI and* ***(D)*** *showing the expression of Nestin (ectodermal marker);* ***(E)*** *A section of EB stained with DAPI and* ***(F)*** *the showing the expression of α-sarcomeric actin (mesodermal marker);* ***(G)*** *A section of EB stained with DAPI and* ***(H)*** *showing the expression of AFP (endodermal marker). Magnification 10x. Scale bar 500.*

The karyotype of hESCs grown with the Tryple culture method for 31 passages was analyzed by Giemsa-banding (G-banding) method. At least 25 individual cells at mitosis were analyzed and 22 cells were found to have a normal 46,XX karyotype (Figure 20A). However, three of the cells had abnormal karyotypes, one having trisomy 5, the other having trisomy 15, and the third having trisomy 16 and tetrasomy X (Figure 20B). There were also few randomly missing chromosomes, for example missing chromosome 11 from the cell that had also trisomy 16 and tetrasomy X.



A B

Figure 20. *The karyotype analysis of hESCs grown with the Tryple culture method for 31 passages.* ***(A)*** *22 cells out of 25 had normal karyotype 46,XX.* ***(B)*** *One cell out of 25 had trisomy 16, tetrasomy X, and missing chromosome 11.*

Because of the small amount of a missing or an extra chromosome in the cells and because there were no similar abnormalities between the cells, these aneuploidies could be considered as artifacts caused by the method. The harsh spreading of the chromosomes at mitosis into the slides might have caused a misplacement of the chromosomes from the mitosis of one cell to the mitosis of another, resulting in missing or extra chromosomes in some cells. However, the tetrasomy X had to be further analyzed by fluorescence *in situ* hybridization (FISH) method. 1142 cells were analyzed by FISH with the X chromosome centromere probes and only three cells were found to have three X chromosomes (0.6 %) and only two cells had four X chromosomes (0.4 %) whereas the rest had the normal two X chromosomes (99 %) (Figure 21). Further confirmation was done for the hESCs grown with the Tryple culture method for 35 passages, using the X chromosome centromere probe and the chromosome 16 centromere probe as a control. The further analysis of 200 individual cells did not reveal any additional abnormalities.

There might occasionally be false missing signals or false positive signals when FISH method is used. The found extra X chromosomes might have been artifacts caused by the FISH method. Although the abnormal karyotypes found for some of the cells might have been real and not artifacts, it could be stated that hESCs grown with the Tryple culture method have normal 46,XX karyotype, because of the low percentage of the abnormal karyotypes. If the found mutations were real they were not clonal and did not give a growth advantage to the cells.

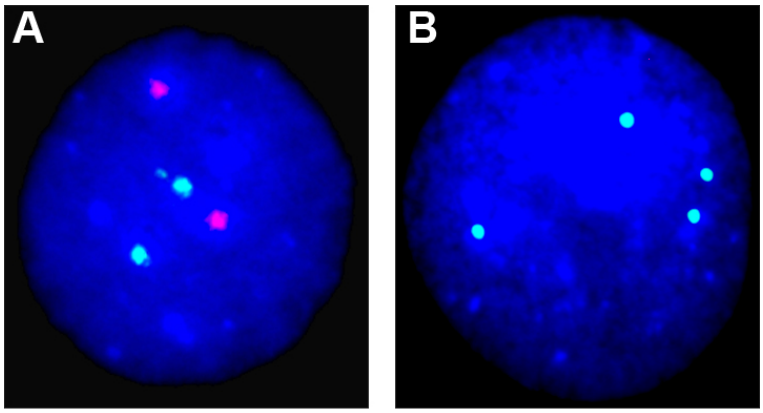


Figure 21. *The FISH analysis of hESCs grown with the Tryple culture method.* ***(A)*** *200 cells out of 200 grown with the Tryple culture method for 35 passages had two X chromosomes and two chromosomes 16;* ***(B)*** *2 cells out of 1142 grown with the Tryple culture method for 31 passages had four X chromosomes.*

5.5. The results of the biomaterial culture method for hESCs

Three different biomaterials (TiO_2, Ti and Zr coated glass pieces from Vivoxid Ltd) were tested for the feeder-free culture of hESCs. Two different media were used in the testing, unconditioned medium (UM) and conditioned medium (CM) from human foreskin fibroblasts (hFFs). The hESCs used in the testing did not attach to any of the biomaterials in the presence of UM, but attached to all biomaterials when CM was used. The hESCs did not attach to the chamber glass used as control.

The hESCs grew on the biomaterials in CM for 4 days, after which they started to detach. The morphology of the colonies grown on the biomaterials was different from the morphology of the colonies grown on feeder cells (Figure 22), therefore the level of differentiation could not be judged according to the morphology. The morphologies of the colonies grown on the three different biomaterials were similar.

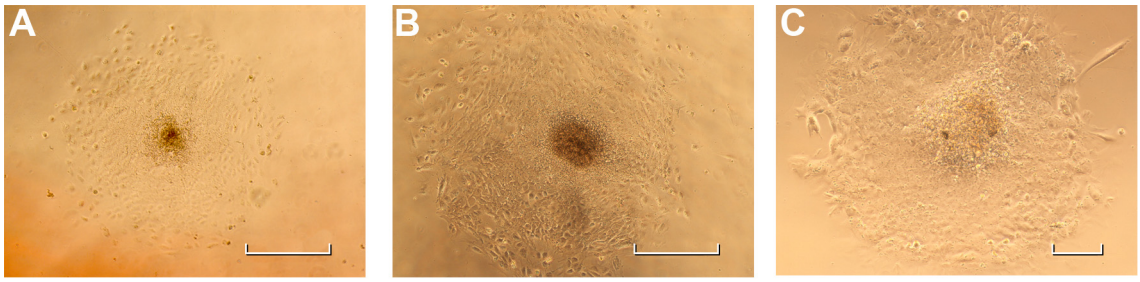


Figure 22. *The morphology of the hESC colonies grown on the tested biomaterials.* ***(A)*** *hESC colony grown on TiO_2 coated glass for 4 after passaging, magnification 4x, scale bar 500 µm;* ***(B)*** *hESC colony grown on Ti coated glass for 4 days after passaging, magnification 4x, scale bar 500 µm;* ***(C)*** *hESC colony grown on Zr coated glass for 2 days after passaging, magnification 10x, scale bar 100 µm.*

The hESCs grown on the biomaterials could not be passaged by conventional methods because they detached easily as single cells and did not attach to the biomaterials anymore. The immunocytochemistry analysis of the hESC colonies on the biomaterials was not successful due to the difficulties in the fixation of the cells as well as in the staining of false positives. The level of differentiation of hESCs grown on the biomaterials was judged after the cells colonies were transferred back on the feeder cells and allowed to proliferate. The morphology of the resulted colonies was similar to undifferentiated colonies grown on feeder cells. Although the hESC colonies expressed a marker common to undifferentiated hESCs (Nanog), they also expressed a marker common to differentiated hESCs (SSEA-1) (Figure 23). The results were consistent with all the tested biomaterials (the data from hESCs grown on TiO_2 and Zr coated glass is not shown).

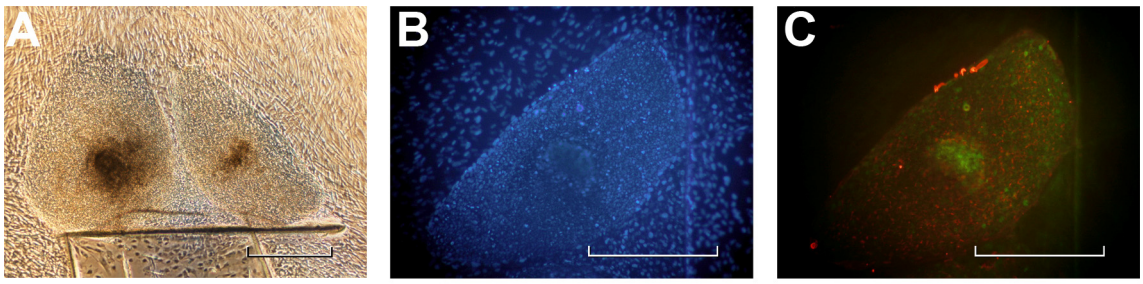


Figure 23. *The morphology of the hESC colonies grown on feeder cells after one passage on Ti coated glass and the expression of a marker common to undifferentiated hESCs (Nanog) and to differentiated hESCs (SSEA-1).* ***(A)*** *hESC colony grown for five days after passaging, magnification 4x,* ***(B)*** *stained with DAPI and* ***(C)*** *showing the expression of Nanog (green) and SSEA-1 (red), magnification 10x. Scale bar 500 μm.*

6. Discussion

6.1. The test bFGF culture method for hESCs

The human recombinant basic fibroblast growth factor (hrbFGF) from Chemicon was selected to the study because it was the only totally animal-free bFGF in the markets in the beginning of this project. The bFGF (from R & D Systems), used as control in this study, was also human recombinant protein but contained small amounts of bovine serum albumine (BSA) and is therefore not suitable for the culture of clinical grade hESCs.

Although undifferentiated hESCs were reached with the test bFGF culture method, proven by immunocytochemistry, the test bFGF did not maintain the undifferentiated growth equally well as the control bFGF. The test bFGF did not promote the expanded growth of hESCs equally well, because the amount of the undifferentiated colonies with all the tested concentrations of test bFGF was significantly smaller than with the control bFGF. However, the test bFGF maintained the undifferentiated growth of hESCs better as the concentration was raised. This may result from the possible rapid degradation of the growth factor in the culture medium; the higher the starting concentration the higher the finishing concentration before medium exchange. Levenstein *et al.* have stated that bFGF is degraded faster in an unconditioned medium (UM) than in a medium conditioned in fibroblast culture, indicating that fibroblasts may excrete some molecules to the medium that stabilizes bFGF (Levenstein *et al.*, 2006). But in this study, human foreskin fibroblasts (hFFs) were used in both the control bFGF culture method and the test bFGF culture method suggesting that the possible FGF stabilizer factor should be present in both culture conditions. However, the added BSA in the control bFGF may have resulted in the differences in the maintenance of undifferentiated growth of hESCs between the control and the test bFGF. It is well known that BSA prevents adhesion of proteins to reaction tubes and stabilizes enzymes during incubation steps. It may also protect purified recombinant proteins from proteolytic degradation by contaminating proteases. Even though the culture medium containing the test bFGF (without BSA) was aliquoted and preserved in +4 °C maximum of one week, and the culture medium for hESCs was changed daily, the degradation of the bFGF might have been too fast. After 24 hours in the +37 °C the amount of bFGF left may have been inadequate to maintain the hESCs undifferentiated. Another possibility is that bFGF had

attached to the tube containing the medium and no or only a small amount of bFGF had been transferred to the cell culture. BSA seems to play a major role in the function of bFGF, but maybe human serum albumine (HSA) could be used instead. HSA could be added to bFGF stocks before freezing, ensuring its preservation after thawing.

The instant growth rates of the undifferentiated colonies grown with the test bFGF culture method were similar to the instant growth rates of the colonies grown with the control bFGF. Although there were no statistically significant differences between the instant growth rates, the undifferentiated colonies grown with the tested bFGF had the same or slightly higher instant growth rates than the colonies grown with the control bFGF. This may be caused by the error in the area measurement of the colonies that does not account the upright growth of the cells. Because the morphology of the hESC colonies grown with the test bFGF was thinner than the morphology of the colonies grown with the control bFGF the instant growth rates of the colonies grown with the test bFGF are probably much lower.

It is reported that the presence or absence of exogenous bFGF does not significantly affect the proliferation rate of hESCs (Dvorak *et al.*, 2005), which could explain that there were no statistically significant difference between the instant growth rates of the colonies grown with the test bFGF and the control bFGF. But this does not explain the morphology differences between the colonies grown with the test bFGF and with the control bFGF. The morphology differences could result only from the differences between the growth factors, because two different cell lines were tested and they both gave the same result and exactly the same culturing conditions were used for the hESCs cultured with the control bFGF and with the test bFGF. Besides the absence or presence of BSA, the growth factors had another difference; their amino acid sequences differ slightly. The control bFGF consists of 157 amino acid residues and its molecular mass is 17,4 kDa (Florkiewicz and Sommer, 1989), whereas the test bFGF consists of 154 amino acids and is 17,2 kDa. The difference is only three amino acids, but it could have a major effect on the proper folding of the protein. The amino acid sequence of the test bFGF is not provided by the manufacturer, so no protein structure comparison could be made. The missing amino acids could be critical for example for the proper folding of the binding site of bFGF that is needed to attach to the receptor and to activate the response pathway. The missing amino acids could have effect also on the folding of the active site of the growth factor, changing the ability of bFGF to produce the needed

signal for the activation of the pathway. The difference could also expose hydrophobic amino acids elsewhere in the protein, making it more vulnerable to proteolysis.

According to the results of this study, hrbFGF from Chemicon is not suitable for hESC culture. The result was unexpected because of the small differences between the two growth factors. This bFGF should be tested again by adding HSA to the protein stock. However, a recent report from Xiao *et al.* revealed that exogenously added bFGF could be replaced by Activin A (Xiao *et al.*, 2006). The medium containing Activin A maintained the hESCs in an undifferentiated state on Matrigel coating without either feeder cells or conditioned medium (CM). It was discovered that Activin A maintains the expression of Oct4 and Nanog ten times higher than bFGF and at lower concentrations also. Only 5 ng/ml of Activin A was sufficient compared to 100 ng/ml of bFGF in the culture of hESCs grown also on Matrigel and UM (Li *et al.*, 2005). The use of Activin A should be tested also in the feeder cell culture of hESCs.

6.2. The SR-3 and X-Vivo 20 culture methods for hESCs

Because the currently used serum replacement (SR) from Gibco Invitrogen contains animal-derived products, several different animal-free SR were tested in our laboratory. SR-3 from Sigma-Aldrich as well as ready-made SR medium, X-Vivo 20, from Cambrex were included into this study. Li *et al.* reported the expansion of hESCs on Matrigel using unconditioned X-Vivo 10 medium from Cambrex (Li *et al.*, 2005). X-Vivo 10 was tested in our laboratory and X-Vivo 20 were chosen to the tests also, although using hFFs as feeder cells.

Neither SR-3 nor X-Vivo 20 were able to maintain hESCs undifferentiated. After the third adaptation phase (1:4, control:test SR) there started to be more differentiated than undifferentiated colonies in the culture. The differentiation stage was judged by the morphology of the colonies and they were divided into three classes; undifferentiated, partly differentiated and differentiated. The evaluation of the differentiation stage is not very accurate, but the result in the last adaptation phase is clear, 100 % of the colonies were completely differentiated. The tested SR and SR-medium may require higher concentrations of bFGF to maintain the undifferentiated growth of hESCs. Li *et al.* used 80 ng/ml of bFGF instead of the 8 ng/ml used in this study *(Li et al.*, 2005). However, the high concentration used in their study might be required because of the feeder-free culture without fibroblasts.

After the adaptation of both SR-3 and X-Vivo 20, the fibroblasts started to detach from the borders of the plate (data not shown). Because feeder cells are an important part of this culture system, this might have a big effect on the differentiation of the hESCs. It is reported that ascorbic acid deficiency in fibroblast culture causes, among other things, an easy disaggregation of the cells from the intracellular matrix by protease (Schafer *et al.*, 1967). The control SR (Ko-SR from Gibco Invitrogen) contains ascorbic acid, but it is not know what the tested SR-3 and X-Vivo 20 contain. They might lack ascorbic acid and the tests should be done again using additional ascorbic acid in the culture medium. The missing or presence of other components in the tested SRs may also have influenced the differentiation of hESCs. It is certain that the ingredients of the control and the test SR and SR medium differ, because once the control SR is entirely replaced, the differentiation of the hESCs is complete. The ingredients should be detected and compared so that the differences could be seen. Ludwig *et al.* reported recently the use of a new animal-free culture medium for hESCs, TeSR1 (Ludwig *et al.*, 2006). They developed the medium based on the ingredients used in the Ko-SR from Gibco Invitrogen, replacing the animal-derived products with human material or recombinant proteins. In addition, they used a combination of collagen IV, fibronectin, laminin and vitronectin coating from human sources instead of feeders or Matrigel. Ludwig *et al.* managed to derive two hESC lines in these animal-free conditions, although only the other maintained stable karyotype. This report is a great step forward in hESC culture, but a great deal of improvement must still be made. The TeSR1 medium contains various ingredients, of which many are used in high concentrations making the medium very expensive. Ludwig *et al.* tested the effect of the absence of several ingredients, and resulted that all of them are needed in the medium, but the results shown in the article are not clear with every ingredient and some of them might be unnecessary for the maintenance of undifferentiated hESCs. Also the purified human matrix components used in the coating are expensive and the data from which the combination was chosen is not shown. Although they found no expression of nonhuman sialic acid, Neu5Gc, in cultured hESCs, the human matrix components provide a potential route of contamination by human pathogens. In addition Ludwig *et al.* used only two different hESC lines and they continued the testing for the cells only three passages. Because of the differences between the hESC lines, the culture method should be tested with several hESC lines and in three passages, the hESCs might not have been adapted to the new medium properly, making these results incomplete.

6.3. The Tryple culture method for hESCs

Tryple Select from Gibco Invitrogen is an animal-free enzyme, which is designed to replace trypsin in the passaging of cells. The possibilities of Tryple for hESC passaging were tested in different ways. Oh *et al.* used an interesting passaging method with collagenase IV (Oh *et al.*, 2005). The collagenase incubation resulted in the pealing off of the colonies, which were collected into a test tube. The colonies were allowed to settle on the bottom of the tube and the resulting supernatant, containing feeder cells and single cells could be discarded. The remaining colonies were pipetted to smaller pieces and then plated. The same method was tested with Tryple, but the colonies did not peal of. However, Tryple detached all the cells from the plate very efficiently (in 1 minute), but even an hour of incubation did not affect the cell-cell interactions.

The hESCs and the feeder cells were collected into a clump, which was tried to pipette into a smaller pieces. The splicing of the clump could be done only with a scalpel, but that resulted in too large pieces. The feeder cells were tried to pull out as one "carpet" at the same time leaving the hESC colonies intact, assuming that the colonies would still be attached to the plate. However, all the colonies detached with the feeder cells. The only way to leave the colonies in the plate and remove the feeders, was to line the colonies with a scalpel before removing the feeders.

The SR used in the hESCs medium may cause the tight cell-cell interactions and make the passaging of the cells difficult. If fibroblasts are cultured in medium containing SR the passaging with trypsin is almost impossible, because the fibroblasts form a similar "carpet" as in the Tryple-testing (unpublished data). Oh *et al.* did not report the medium they used, so it is possible that their passaging method is possible only in a serum containing culture (Oh *et al.*, 2005).

The Tryple passaging method was as laborious as the original mechanical passaging and the same skills were required for both methods. However, it was easier to detach the scalpeld pieces from the plate after Tryple treatment. In mechanical passaging, the pieces are sometimes hard to detach with a needle, which results in torn pieces that do not easily attach to new feeder plates. It was also easier to detach smaller pieces after Tryple treatment than with original mechanical passaging, which can result in more expanded growth of hESCs.

The Tryple culture method maintained the undifferentiated growth of hESCs, proven by immunocytochemistry analysis, and the amount of undifferentiated and partly

differentiated colonies was similar with the control culture method, allowing the expanded growth of the hESCs similarly as the control method. The instant growth rates between the hESC colonies grown with the control culture method and with the Tryple culture method did not have statistically significant differences. The hESCs cultured with Tryple method remained pluripotent, which is shown from the differentiation of the embryoid bodies into all three embryonic germ layers. The results of the RT-PCR analysis indicates that the hESCs grown with the Tryple culture method preferred to differentiate towards the cells representing the ectoderm layer. These cells could be more easily differentiated for example to neurons.

The enzymatic passaging method may cause karyotypic chances in hESCs, especially trisomy 12 and/or 17 (Draper *et al.*, 2004b; Mitalipova *et al.*, 2005). The mutations may result from the harsh enzyme treatment that affects all the cells in the culture plate, whereas mechanical passaging stresses only the cells that are in the borders of the passaged piece of a cell colony. On the other hand, the mutations may result from the passaging method and not from the enzyme itself. There are often a lot of single cells after the enzymatic passaging of the cells and it is known that hESCs prefer the cell-cell contacts. Proliferation from a single cell may cause pressure to the cell and it might need chromosomal mutations to survive. Although the Tryple culture method did not allow the cells to detach as single cells, an enzyme was used for the passaging and therefore the analyzing of the karyotype of these cells was important.

The karyotype analysis of hESCs grown with the Tryple culture method was performed by Giemsa-banding (G-banding) and fluorescence *in situ* hybridization (FISH) method by Laboratoriokeskus Ltd, where these methods for hESCs are currently optimized. Twenty-two cells out of 25 were found to have normal 46,XX karyotype by G-banding method whereas three of these cells had abnormal karyotypes. These found abnormalities were all different from each other and none of them were the most commonly occurred mutations found from the hESCs (neither trisomy 12 nor trisomy 17). One of these found mutations was tetrasomy X, where two extra X chromosomes were present in the cell. This possible mutation was further analyzed by FISH from the cells grown with the Tryple culture method for 31 passages, which revealed only three cells with one extra X chromosome and only two cells with two extra X chromosomes out of 1142 cells examined. In this experiment, only the centromere probes for X chromosomes were used and no other probes for different chromosome as a control were used. This caused uncertainty in the interpretation of the results, because it could not be

determined that were there only extra X chromosomes or was the entire genome of the cell triploid or tetraploid. The hESCs grown with the Tryple culture method for 35 passages were further analyzed by FISH using X chromosome centromere probe and the chromosome 16 centromere probe as a control. These additional 200 individual cells were examined and no abnormalities were found.

The percentage of the abnormal karyotypes found is so low that they might be artifacts caused by the methods used. The G-banding and FISH method for hESCs were not yet fully optimized in Laboratoriokeskus Ltd at the time of this study and the occurrence of artifacts was quite probable. Although the found abnormalities would have been real and not artifacts, the mutations were not clonal and did not gave the cells any growth advantage, because no similarly mutated cells were found with G-banding. These mutations would probably be left off from the later passages. It can be stated that the hESCs grown with the Tryple culture method have normal 46,XX karyotype even after 31 passages. The common karyotypic changes caused by the enzymatic passaging methods usually occur after extended culture of hESCs. Mitalipova *et al.* found karyotypic changes after 23 passages when cell dissociation buffer was used and after 25 passages after collagenase or trypsin were used for passaging (Mitalipova *et al.*, 2005). The results of this study indicate that Tryple culture method might maintain stable karyotype better than the methods were trypsin, collagenase or cell dissociation buffer is used. These results might also reinforce the theory that karyotypic mutations occur if the cells are passaged as single cells and that the enzyme itself does not cause the mutations.

The karyotype of the cells grown with the Tryple culture method must be analyzed regularly to make sure that no changes will occur later and that the possible mutations found now are not multiplied in the later passages. The karyotype of the HS181 cells used as control cells must also be analyzed so that the effect of Tryple culture method can be shown and the effect of the age of the cell line can be left out. In conclusion, the Tryple culture method can be recommended for the expansion of hESCs and it is a good choice for passaging hESCs, especially cell lines with thin morphologies.

6.4. The biomaterial culture method for hESCs

Three different biomaterials were tested in cooperation with Vivoxid Ltd from Turku. Because titania coatings in implants results in good soft tissue attachment (Areva *et al.*, 2004), the coating was decided to test for hESC attachment. Besides titania (TiO2) testing, also titanium (Ti) and zirconium (Zr) coatings were tested because they have similar properties with titania. 10 mm x 10 mm glass pieces were coated in a manner described in Areva *et al.* 2004, and placed on a culture chamber. Human ESCs did not attach to the biomaterials in the presence of normal hES-medium (unconditioned medium), but cells attached to all the biomaterials in conditioned hES-medium similarly as reported with other feeder-free methods such as Matrigel (Xu *et al.*, 2001). Feeder cells must secrete some molecules that assist this attachment. The pieces of the hESC colonies did not attach to culture chamber glass used as control, indicating that the coatings have also some effect on the attachment. The percentage values of the attachment was not able to record because the testing was difficult, mainly because the small size of the biomaterials. The culture chamber was twice as big as the biomaterial and only a small bump was needed to dislocate the cell clumps from the biomaterials before the attachment.

The cell clumps that attached proliferated for four days, after which the cells started to detach. The morphology of the colonies was totally different from those grown on feeder cells, but because the feeder layer influences the shape of the colonies a lot, no conclusions about the differentiation stage could be made. The morphology of the colonies grown on the biomaterials resembles that grown on fibronectin matrix (Amit *et al.*, 2004), but they were less compact. The cells were tried to analyze with immunocytochemistry, but the fixation of the cells was difficult and when the fixation worked the immunostaining gave false positive results. New methods for fixation and for immunostaining of these cells should be developed and optimized.

The passaging of the cells grown on the biomaterials was impossible with the techniques in hand. The cells detached easily as single cells and they did not attach to the biomaterials after that. A new passaging method should be created in order to test the ability of the biomaterials to maintain the growth of the cells. The analysis of the differentiation stage of the hESCs grown on the biomaterials was done by transferring the cells back on feeder cells. The colonies grown on the biomaterials for one passage, were detached in as big piece as possible and the pieces were then transferred back on

feeders and allowed to proliferate for five days. The morphology of the resulting colonies was similar to undifferentiated colonies grown on feeder cells, but the immunocytochemistry analysis of the colonies showed the expression of SSEA-1 indicating the presence of differentiated hESCs. There were also some cells positive to Nanog (expressed in undifferentiated hESCs), but because the cells were maintained only one passage on the biomaterials, the undifferentiated cells may have preserved on the biomaterials in the piece, which was transferred to the biomaterials.

No finite conclusions can be made from the convenience of these biomaterials to the culture of hESCs. New methods should be optimized and a large scale testing would be needed. The size of the biomaterial should be bigger and the best choice would be a culture plate coated with these materials. No differences between the three materials were observed in this small scale testing and further testing is required.

7. Conclusions

One of the aims of this study, and perhaps the most important one, was to optimize culturing conditions for human embryonic stem cells (hESCs) towards animal-free conditions. Human recombinant basic fibroblast growth factor (hrbFGF) from Chemicon, serum replacement SR-3 from Sigma-Aldrich and serum replacement medium X-Vivo 20 from Cambrex were tested for this purpose. All of these tested components failed to maintain the growth of undifferentiated hESCs better than the control conditions. Although this aim was not reached in this study, a lot of valuable information was gained and a direction of new testing was accomplished.

Development of an easier way for hESC passaging was the second aim of the study. No universally easier way was found, but based on the results and the experience from the Tryple culture method, it is useful in passaging hESC colonies with thin morphologies and it could be used to result in faster expanded growth of hESCs than the conventional mechanical passaging. In addition, the hESCs grown with the Tryple culture method for 31 passages had normal 46,XX karyotype making Tryple enzyme a better option for the currently used enzymes.

The tested biomaterials could not yet be used to maintain the undifferentiated growth of hESCs instead of feeder cells. Although this aim also remains to be reach, the study raised several questions about biomaterials and their potential. Biomaterials could be the perfect choice for feeder cells, but before it can be proofed, the factors maintaining the undifferentiated growth of hESCs must be better determined.

8. References

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002). Molecular biology of the cell, Fourth edn: Garland Science, p. 1262.

Amit, M., Carpenter, M. K., Inokuma, M. S., Chiu, C. P., Harris, C. P., Waknitz, M. A., Itskovitz-Eldor, J., and Thomson, J. A. (2000). Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol *227*, 271-278.

Amit, M., Margulets, V., Segev, H., Shariki, K., Laevsky, I., Coleman, R., and Itskovitz-Eldor, J. (2003). Human feeder layers for human embryonic stem cells. Biol Reprod *68*, 2150-2156.

Amit, M., Shariki, C., Margulets, V., and Itskovitz-Eldor, J. (2004). Feeder layer- and serum-free culture of human embryonic stem cells. Biol Reprod *70*, 837-845.

Amit, M., Winkler, M. E., Menke, S., Bruning, E., Buscher, K., Denner, J., Haverich, A., Itskovitz-Eldor, J., and Martin, U. (2005). No evidence for infection of human embryonic stem cells by feeder cell-derived murine leukemia viruses. Stem Cells *23*, 761-771.

Areva, S., Paldan, H., Peltola, T., Narhi, T., Jokinen, M., and Linden, M. (2004). Use of sol-gel-derived titania coating for direct soft tissue attachment. J Biomed Mater Res A *70*, 169-178.

Asahara, T., Kalka, C., and Isner, J. M. (2000). Stem cell therapy and gene transfer for regeneration. Gene Ther *7*, 451-457.

Battista, S., Guarnieri, D., Borselli, C., Zeppetelli, S., Borzacchiello, A., Mayol, L., Gerbasio, D., Keene, D. R., Ambrosio, L., and Netti, P. A. (2005). The effect of matrix composition of 3D constructs on embryonic stem cell differentiation. Biomaterials *26*, 6194-6207.

Bradley, J. A., Bolton, E. M., and Pedersen, R. A. (2002). Stem cell medicine encounters the immune system. Nat Rev Immunol *2*, 859-871.

Buzzard, J. J., Gough, N. M., Crook, J. M., and Colman, A. (2004). Karyotype of human ES cells during extended culture. Nat Biotechnol *22*, 381-382; author reply 382.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., and Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell *113*, 643-655.

Cheng, L., Hammond, H., Ye, Z., Zhan, X., and Dravid, G. (2003). Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture. Stem Cells *21*, 131-142.

Cibelli, J. B., Stice, S. L., Golueke, P. J., Kane, J. J., Jerry, J., Blackwell, C., Ponce de Leon, F. A., and Robl, J. M. (1998). Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells. Nat Biotechnol *16*, 642-646.

Clarke, D., and Frisen, J. (2001). Differentiation potential of adult stem cells. Curr Opin Genet Dev *11*, 575-580.

Clarke, D. L., Johansson, C. B., Wilbertz, J., Veress, B., Nilsson, E., Karlstrom, H., Lendahl, U., and Frisen, J. (2000). Generalized potential of adult neural stem cells. Science *288*, 1660-1663.

Draper, J. S., Moore, H. D., Ruban, L. N., Gokhale, P. J., and Andrews, P. W. (2004a). Culture and characterization of human embryonic stem cells. Stem Cells Dev *13*, 325-336.

Draper, J. S., Smith, K., Gokhale, P., Moore, H. D., Maltby, E., Johnson, J., Meisner, L., Zwaka, T. P., Thomson, J. A., and Andrews, P. W. (2004b). Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nat Biotechnol *22*, 53-54.

Drukker, M., Katchman, H., Katz, G., Even-Tov Friedman, S., Shezen, E., Hornstein, E., Mandelboim, O., Reisner, Y., and Benvenisty, N. (2006). Human embryonic stem cells and their differentiated derivatives are less susceptible for immune rejection than adult cells. Stem Cells *24*, 221-229.

Dvorak, P., Dvorakova, D., Koskova, S., Vodinska, M., Najvirtova, M., Krekac, D., and Hampl, A. (2005). Expression and potential role of fibroblast growth factor 2 and its receptors in human embryonic stem cells. Stem Cells *23*, 1200-1211.

Faulkner, J., and Keirstead, H. S. (2005). Human embryonic stem cell-derived oligodendrocyte progenitors for the treatment of spinal cord injury. Transpl Immunol *15*, 131-142.

Ferrari, G., Cusella-De Angelis, G., Coletta, M., Paolucci, E., Stornaiuolo, A., Cossu, G., and Mavilio, F. (1998). Muscle regeneration by bone marrow-derived myogenic progenitors. Science *279*, 1528-1530.

Florkiewicz, R. Z., and Sommer, A. (1989). Human basic fibroblast growth factor gene encodes four polypeptides: three initiate translation from non-AUG codons. Proc Natl Acad Sci U S A *86*, 3978-3981.

Frisen, J. (2002). Stem cell plasticity? Neuron *35*, 415-418.

Gerecht-Nir, S., Cohen, S., Ziskind, A., and Itskovitz-Eldor, J. (2004). Three-dimensional porous alginate scaffolds provide a conducive environment for generation of well-vascularized embryoid bodies from human embryonic stem cells. Biotechnol Bioeng *88*, 313-320.

Healy, L., Hunt, C., Young, L., and Stacey, G. (2005). The UK Stem Cell Bank: its role as a public research resource centre providing access to well-characterised seed stocks of human stem cell lines. Adv Drug Deliv Rev *57*, 1981-1988.

Heins, N., Lindahl, A., Karlsson, U., Rehnstrom, M., Caisander, G., Emanuelsson, K., Hanson, C., Semb, H., Bjorquist, P., Sartipy, P., and Hyllner, J. (2006). Clonal derivation and characterization of human embryonic stem cell lines. J Biotechnol *122*, 511-520.

Henderson, J. K., Draper, J. S., Baillie, H. S., Fishel, S., Thomson, J. A., Moore, H., and Andrews, P. W. (2002). Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. Stem Cells *20*, 329-337.

Hoffman, L. M., and Carpenter, M. K. (2005). Characterization and culture of human embryonic stem cells. Nat Biotechnol *23*, 699-708.

Hovatta, O., Mikkola, M., Gertow, K., Stromberg, A. M., Inzunza, J., Hreinsson, J., Rozell, B., Blennow, E., Andang, M., and Ahrlund-Richter, L. (2003). A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. Hum Reprod *18*, 1404-1409.

Hwang, W. S., Roh, S. I., Lee, B. C., Kang, S. K., Kwon, D. K., Kim, S., Kim, S. J., Park, S. W., Kwon, H. S., Lee, C. K.*, et al.* (2005). Patient-specific embryonic stem cells derived from human SCNT blastocysts. Science *308*, 1777-1783.

Hwang, W. S., Ryu, Y. J., Park, J. H., Park, E. S., Lee, E. G., Koo, J. M., Jeon, H. Y., Lee, B. C., Kang, S. K., Kim, S. J.*, et al.* (2004). Evidence of a pluripotent human embryonic stem cell line derived from a cloned blastocyst. Science *303*, 1669-1674.

Hyslop, L., Stojkovic, M., Armstrong, L., Walter, T., Stojkovic, P., Przyborski, S., Herbert, M., Murdoch, A., Strachan, T., and Lako, M. (2005). Downregulation of NANOG induces differentiation of human embryonic stem cells to extraembryonic lineages. Stem Cells *23*, 1035-1043.

Inzunza, J., Gertow, K., Stromberg, M. A., Matilainen, E., Blennow, E., Skottman, H., Wolbank, S., Ahrlund-Richter, L., and Hovatta, O. (2005). Derivation of human embryonic stem cell lines in serum replacement medium using postnatal human fibroblasts as feeder cells. Stem Cells *23*, 544-549.

Inzunza, J., Sahlen, S., Holmberg, K., Stromberg, A. M., Teerijoki, H., Blennow, E., Hovatta, O., and Malmgren, H. (2004). Comparative genomic hybridization and karyotyping of human embryonic stem cells reveals the occurrence of an isodicentric X chromosome after long-term cultivation. Mol Hum Reprod *10*, 461-466.

Itskovitz-Eldor, J., Schuldiner, M., Karsenti, D., Eden, A., Yanuka, O., Amit, M., Soreq, H., and Benvenisty, N. (2000). Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol Med *6*, 88-95.

Keller, G., and Snodgrass, H. R. (1999). Human embryonic stem cells: the future is now. Nat Med *5*, 151-152.

Khoo, M. L., McQuade, L. R., Smith, M. S., Lees, J. G., Sidhu, K. S., and Tuch, B. E. (2005). Growth and differentiation of embryoid bodies derived from human embryonic stem cells: effect of glucose and basic fibroblast growth factor. Biol Reprod *73*, 1147-1156.

Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L., and Shay, J. W. (1994). Specific association of human telomerase activity with immortal cells and cancer. Science *266*, 2011-2015.

Kim, S. J., Cheon, S. H., Yoo, S. J., Kwon, J., Park, J. H., Kim, C. G., Rhee, K., You, S., Lee, J. Y., Roh, S. I., and Yoon, H. S. (2005). Contribution of the PI3K/Akt/PKB signal pathway to maintenance of self-renewal in human embryonic stem cells. FEBS Lett *579*, 534-540.

Koivisto, H., Hyvärinen, M., Strömberg, A.-M., Inzunza, J., Matilainen, E., Mikkola, M., Hovatta, O., and Teerijoki, H. (2004). Cultures of human embryonic stem cells - serum replacement medium or serum-containing media and the effect of basic fibrolast growth factor. Reproductive BioMedicine Online *9*, 330-337.

Levenberg, S., Huang, N. F., Lavik, E., Rogers, A. B., Itskovitz-Eldor, J., and Langer, R. (2003). Differentiation of human embryonic stem cells on three-dimensional polymer scaffolds. Proc Natl Acad Sci U S A *100*, 12741-12746.

Levenstein, M. E., Ludwig, T. E., Xu, R. H., Llanas, R. A., Vandenheuvel-Kramer, K., Manning, D., and Thomson, J. A. (2006). Basic fibroblast growth factor support of human embryonic stem cell self-renewal. Stem Cells *24*, 568-574.

Li, Y., Powell, S., Brunette, E., Lebkowski, J., and Mandalam, R. (2005). Expansion of human embryonic stem cells in defined serum-free medium devoid of animal-derived products. Biotechnol Bioeng *91*, 688-698.

Ludwig, T. E., Levenstein, M. E., Jones, J. M., Berggren, W. T., Mitchen, E. R., Frane, J. L., Crandall, L. J., Daigh, C. A., Conard, K. R., Piekarczyk, M. S.*, et al.* (2006). Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol *24*, 185-187.

Lumelsky, N., Blondel, O., Laeng, P., Velasco, I., Ravin, R., and McKay, R. (2001). Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science *292*, 1389-1394.

Maitra, A., Arking, D. E., Shivapurkar, N., Ikeda, M., Stastny, V., Kassauei, K., Sui, G., Cutler, D. J., Liu, Y., Brimble, S. N.*, et al.* (2005). Genomic alterations in cultured human embryonic stem cells. Nat Genet *37*, 1099-1103.

Martin, M. J., Muotri, A., Gage, F., and Varki, A. (2005). Human embryonic stem cells express an immunigenic nonhuman sialic acid. Nature Medicine *11*, 1-5.

Matthiessen-Guyader (July 2004). Survey on opinions from National Ethics Committees or similar bodies, public debate and national legislation in relation to human embryonic stem cell research and use, Vol. 1 (EU member states). *http://europa.eu.int/comm/research/biosociety/pdf/mb_states_230804.pdf*, Data visited in 13 February 2006.

Mitalipova, M. M., Rao, R. R., Hoyer, D. M., Johnson, J. A., Meisner, L. F., Jones, K. L., Dalton, S., and Stice, S. L. (2005). Preserving the genetic integrity of human embryonic stem cells. Nat Biotechnol *23*, 19-20.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H., and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell *95*, 379-391.

Nistor, G. I., Totoiu, M. O., Haque, N., Carpenter, M. K., and Keirstead, H. S. (2005). Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation. Glia *49*, 385-396.

Odorico, J. S., Kaufman, D. S., and Thomson, J. A. (2001). Multilineage differentiation from human embryonic stem cell lines. Stem Cells *19*, 193-204.

Oh, S. K., Kim, H. S., Park, Y. B., Seol, H. W., Kim, Y. Y., Cho, M. S., Ku, S. Y., Choi, Y. M., Kim, D. W., and Moon, S. Y. (2005). Methods for expansion of human embryonic stem cells. Stem Cells *23*, 605-609.

Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S. M., Li, B., Pickel, J., McKay, R., Nadal-Ginard, B., Bodine, D. M.*, et al.* (2001). Bone marrow cells regenerate infarcted myocardium. Nature *410*, 701-705.

Passier, R., Oostwaard, D. W., Snapper, J., Kloots, J., Hassink, R. J., Kuijk, E., Roelen, B., de la Riviere, A. B., and Mummery, C. (2005). Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free cultures. Stem Cells *23*, 772-780.

Paul, G., Li, J. Y., and Brundin, P. (2002). Stem cells: hype or hope? Drug Discov Today *7*, 295-302.

Reubinoff, B. E., Pera, M. F., Fong, C. Y., Trounson, A., and Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol *18*, 399-404.

Richards, M., Fong, C. Y., Chan, W. K., Wong, P. C., and Bongso, A. (2002). Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nat Biotechnol *20*, 933-936.

Richards, M., Tan, S., Fong, C. Y., Biswas, A., Chan, W. K., and Bongso, A. (2003). Comparative evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem cells. Stem Cells *21*, 546-556.

Rodriquez, C. I., Galán, A., Valbuena, D., and Simón, C. (2006). Derivation of clinical-grade human embryonic stem cells. Reproductive BioMedicine Online *12*, 112-118.

Rolletschek, A., Blyszczuk, P., and Wobus, A. M. (2004). Embryonic stem cell-derived cardiac, neuronal and pancreatic cells as model systems to study toxicological effects. Toxicol Lett *149*, 361-369.

Sato, N., Sanjuan, I. M., Heke, M., Uchida, M., Naef, F., and Brivanlou, A. H. (2003). Molecular signature of human embryonic stem cells and its comparison with the mouse. Dev Biol *260*, 404-413.

Schafer, I. A., Silverman, L., Sullivan, J. C., and Robertson, W. V. (1967). Ascorbic acid deficiency in cultured human fibroblasts. J Cell Biol *34*, 83-95.

Schuldiner, M., Yanuka, O., Itskovitz-Eldor, J., Melton, D. A., and Benvenisty, N. (2000). Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A *97*, 11307-11312.

Schulz, T. C., Palmarini, G. M., Noggle, S. A., Weiler, D. A., Mitalipova, M. M., and Condie, B. G. (2003). Directed neuronal differentiation of human embryonic stem cells. BMC Neurosci *4*, 27.

Seiler, A., Visan, A., Buesen, R., Genschow, E., and Spielmann, H. (2004). Improvement of an in vitro stem cell assay for developmental toxicity: the use of molecular endpoints in the embryonic stem cell test. Reprod Toxicol *18*, 231-240.

Skottman, H., Mikkola, M., Lundin, K., Olsson, C., Strömberg, A.-M., Tuuri, T., Otonkoski, O., Hovatta, O., and Lahesmaa, R. (2005). Gene expression signatures of seven individual human embryonic stem cell lines. Stem Cells *23*, 1343-1356.

Skottman, H., Stromberg, A. M., Matilainen, E., Inzunza, J., Hovatta, O., and Lahesmaa, R. (2006). Unique gene expression signature by human embryonic stem cells cultured under serum free conditions correlates with their enhanced and prolonged growth in an undifferentiated stage. Stem Cells *24*, 151-167.

Stojkovic, M., Lako, M., Stojkovic, P., Stewart, R., Przyborski, S., Armstrong, L., Evans, J., Herbert, M., Hyslop, L., Ahmad, S.*, et al.* (2004). Derivation of human embryonic stem cells from day-8 blastocysts recovered after three-step in vitro culture. Stem Cells *22*, 790-797.

Stojkovic, P., Lako, M., Przyborski, S., Stewart, R., Armstrong, L., Evans, J., Zhang, X., and Stojkovic, M. (2005). Human-serum matrix supports undifferentiated growth of human embryonic stem cells. Stem Cells *23*, 895-902.

TENK, ETENE, TUKIJA, KYTÖ, BTNK, and GTLK (2003). Ihmisen kantasolut, kloonaus ja tutkimus (Vantaa: Kirjapaino Keili Oy).

Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D. M., Nakano, Y., Meyer, E. M., Morel, L., Petersen, B. E., and Scott, E. W. (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature *416*, 542-545.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. Science *282*, 1145-1147.

Thomson, J. A., Kalishman, J., Golos, T. G., Durning, M., Harris, C. P., Becker, R. A., and Hearn, J. P. (1995). Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci U S A *92*, 7844-7848.

Thomson, J. A., and Odorico, J. S. (2000). Human embryonic stem cell and embryonic germ cell lines. Trends Biotechnol *18*, 53-57.

Xiao, L., Yuan, X., and Sharkis, S. J. (2006). Activin A maintains self-renewal and regulates FGF, Wnt and BMP pathways in human embryonic stem cells. Stem Cells Express [Epub February 2, 2006; doi:10.1634/stemcells.2005-0299].

Xie, C. Q., Lin, G., Yuan, D., Wang, J., Liu, T. C., and Lu, G. X. (2005). Proliferative feeder cells support prolonged expansion of human embryonic stem cells. Cell Biol Int *29*, 623-628.

Xu, C., Inokuma, M. S., Denham, J., Golds, K., Kundu, P., Gold, J. D., and Carpenter, M. K. (2001). Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol *19*, 971-974.

Xu, C., Jiang, J., Sottile, V., McWhir, J., Lebkowski, J., and Carpenter, M. K. (2004). Immortalized fibroblast-like cells derived from human embryonic stem cells support undifferentiated cell growth. Stem Cells *22*, 972-980.

Xu, R. H., Peck, R. M., Li, D. S., Feng, X., Ludwig, T., and Thomson, J. A. (2005). Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Methods *2*, 185-190.

Ying, Q. L., Nichols, J., Evans, E. P., and Smith, A. G. (2002). Changing potency by spontaneous fusion. Nature *416*, 545-548.